How flower biology and breeding system affect the reproductive success of the narrow endemic *Polygala vayredae* Costa (Polygalaceae)

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Polygala vayredae is a narrow endemic species from the oriental pre-Pyrenees. Despite its conservation status and rarity, no information is available on its reproductive biology. As the flower is the structure directly involved in pollinator attraction, its morphological and functional traits have major effects on the reproductive success of the plant. In this work, the flower biology and breeding system of *P. vayredae* were studied to evaluate how they affect the reproductive outcome in natural populations. Flower morphology, flower rewards, and male and female functioning throughout the lifespan of the flower were assessed. Pollination experiments, involving pollinator exclusion and pollen from different sources, were conducted, and the pollen ovule index was determined. Female fitness and the occurrence of pollen limitation were assessed in three natural populations over 2 years by observing the presence of pollen on the stigma, pollen tube development, and fruit production. *Polygala vayredae* flowers are elaborate and long-lived with nectar rewards. The floral traits are well adapted to xenogamy and entomophily, which are in accordance with the observed breeding system and auto-incompatibility system. No mechanism of reproductive assurance was observed and *P. vayredae* strictly depends on pollinators to set fruit. Low fruit production was observed in the studied populations, which was largely the result of scarce, unreliable, and/or inefficient pollinators and poor pollen quality. In addition, available resources may be a limiting factor. The reproductive strategy of *P. vayredae* prevents inbreeding depression by a self-incompatibility system, which in years of scarce pollinators is overcome by the plant habit. © 2008 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2008, **157**, 67–81.

ADDITIONAL KEYWORDS: endemic species – pollen limitation – reproductive system – secondary pollen presentation.

INTRODUCTION

The reproductive pattern is one of the key factors leading to the abundance, distribution, and genetic diversity of organisms. In flowering plants, a highly diverse array of floral traits and reproductive systems have evolved, varying from obligated cross-fertilization to obligated or promoted self-fertilization, with each strategy presenting selective advantages and disadvantages (reviewed by Takebayashi &

Morrell, 2001). In rare, endemic, and/or narrow species, these patterns assume special importance as they will operate in a reduced number of individuals/populations, primarily determining their reproductive success and secondarily affecting their population dynamics and genetic diversity. Although selfing presents selective advantages, such as reproductive assurance (during colonization processes, bottlenecks, and scarce or unreliable pollinators) and twofold transmission of genes, crossing increases genetic diversity reducing the risk of inbreeding depression. Therefore, it is important to search for distinct

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biological attributes of narrow endemic plants. For example, Murray *et al*. (2002) observed that, at a spatial scale, these species generally presented shorter flowering periods, and Lavergne *et al*. (2004) reported fewer and smaller flowers, less stigma–anther separation, and lower pollen/ovule (P/O) ratios in narrow endemic species. Moreover, it is generally considered that these species harbour significantly lower genetic diversity at the population level when compared with their widespread congeners (for example, Gitzendanner & Soltis, 2000; Hannan & Orick, 2000; Cole, 2003). This pattern does not seem to result from a predominant autogamous system within rare species, as it was independently observed in both self-compatible and self-incompatible species (Hamrick & Godt, 1996), but is probably the consequence of a reduced population size (Cole, 2003). Nevertheless, endemism and rarity phenomena result from a diverse array of evolutionary scenarios and interactions, making it difficult to identify the effect of evolutionary patterns on reproductive strategies.

The genus *Polygala* L. (Polygalaceae) comprises about 725 species distributed around the world, and presents a marked pattern of regional endemism and a significant representation in diversification centres, as defined by the International Union for Conservation of Nature (IUCN) and World Wide Fund for Nature WWF programme (Paiva, 1998). Despite the conservation importance of this genus, few studies have been performed so far and the information available on its reproductive ecology is scarce. Several reproductive strategies have been described or assumed for *Polygala* species. In general, individuals are described as entomophilous with specialized flower morphology (Faegri & van der Pijl, 1979; Brantjes, 1982), elaborate mechanisms of secondary pollen presentation (Ladd & Donaldson, 1993; Yeo, 1993; Paiva, 1998), and flower ecology and pollination mechanisms parallel with those observed in Fabaceae (Brantjes & van der Pijl, 1980; Westerkamp, 1999). Nevertheless, as a result of androecia and gynoecia morphology and disposition (see figures in Paiva, 1998), auto-pollination is assumed to be the most common phenomenon (Venkatesh, 1955, 1956; Dube, 1962; Miller, 1971), frequently occurring by delayed selfing (Lack & Kay, 1987; Norderhaug, 1995; Weekley & Brothers, 2006). In addition, several species produce chasmogamous and cleistogamous flowers, ensuring sexual reproduction and the possibility for crossing (James, 1957; Ferrara & Quinn, 1985, 1986; Weekley & Brothers, 2006). These different reproductive strategies will result in different outputs that directly affect the population dynamics, with their understanding being of major importance for the assessment of the status of rare species and the development of management strategies (Schemske *et al*., 1994).
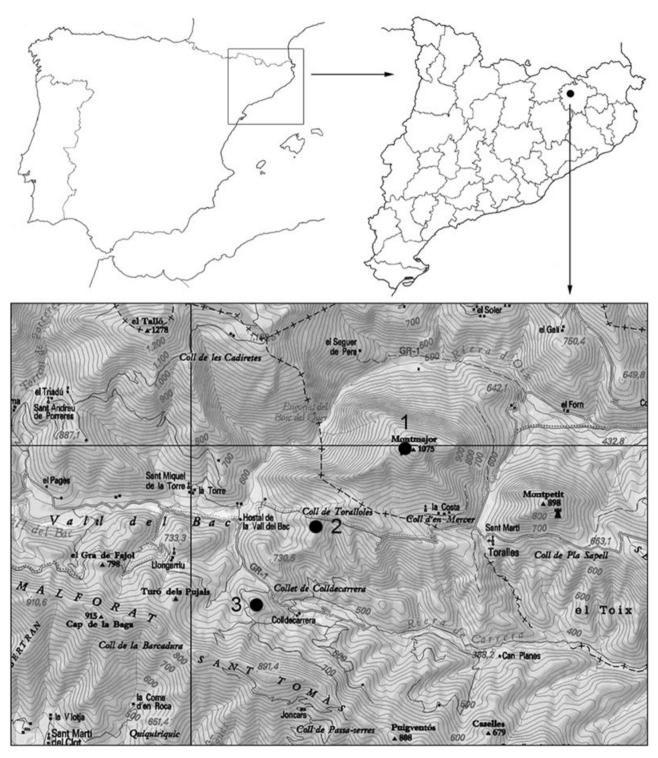
Polygala vayredae Costa (Polygalaceae) is a narrow endemic species from the oriental pre-Pyrenees, present only in an area of approximately 12 km². According to the IUCN categories, a preliminary endangered status has been given ('vulnerable'; VV. AA., 2000), and it is hypothesized that this species is a relict that survived through the Quaternary glaciations in restricted habitat pockets, where it currently occurs. Despite its conservation status, rarity, and location in a protected area (Espai d'interès Natural de l'Alta Garrotxa, Catalunya, Spain), no information is available on the reproductive biology of *P. vayredae*.

In this work, the flower biology and breeding system of *P. vayredae* were studied to evaluate how they affect the reproductive outcome in natural populations. Three questions emerged: (1) How does the flower function as a sexual reproductive entity?; (2) Does the plant depend on pollinators and/or present an alternative mechanism for reproductive assurance?; (3) What are the consequences of this on the reproductive success of natural populations? The flower morphology and floral rewards (both directly involved in plant–animal interactions) were studied, and the male and female functioning throughout the lifespan of the flower was examined, in order to evaluate the occurrence of mechanisms that prevent or open the possibility for selfing. Pollination experiments, involving pollinator exclusion and pollen from different sources, were conducted, and the pollen ovule index was determined. Female fitness and the occurrence of pollen limitation were assessed in three natural populations over 2 years by observing the presence of pollen on the stigma, pollen tube development, and fruit production. This constitutes the first study on the flower biology and reproduction of *P. vayredae*. With this study, we intend to contribute to the knowledge of the reproductive strategies in the genus *Polygala*, and to provide relevant information that is essential for the correct management and conservation of *P. vayredae*.
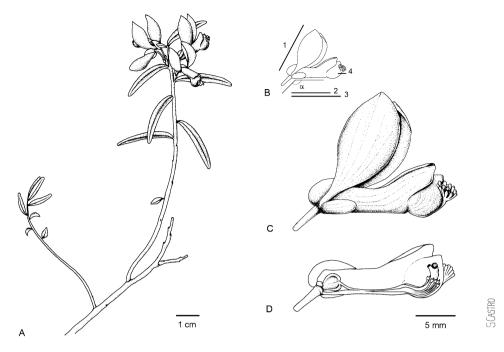
MATERIAL AND METHODS
PLANT AND STUDY AREA

Polygala vayredae is a small shrublet, annually sending up shoots from a rootstock that forms dense tufts. This species presents an early flowering period (April to May) and appears in mesophytic and xeric meadows (*Mesobromion*), with *Pinus sylvestris* and *Buxus sempervirens*, and in rocky places (*Saxifragion mediae*), mainly under the *Quercetum pubescentis* domain. *Polygala vayredae* occurs in an area of approximately 12 km² in Alta Garrotxa, Girona (Catalunya, Spain; UTM DG57 and DG58; Fig. 1). The largest populations are observed in pine forests,

Figure 1. Distribution of *Polygala vayredae* and location of the studied populations (1, Montmajor; 2, Serrat dels Boixos; 3, Colldecarrera) (regional topographic map, 1 : 50 000) in Alta Garrotxa, Catalunya, Spain (Iberian Peninsula).

and these sites constitute the most suitable habitat for its development (Bolòs, 1946); smaller populations usually occur in sunny rocky places. In this narrow distribution area, three populations were selected: (1) Montmajor, 1070 m, exposed rocky slopes; (2) Serrat dels Boixos, 750 m, open pine forest; and (3) Colldecarrera, 630 m, open pine forest (Fig. 1). Flower samples (preserved in ethanol 70%) and reproductive

Figure 2. *Polygala vayredae*: A, habit; B, morphometric measures performed in the flowers [angle of flower presentation (α) and length of wing-sepals (1), corolla tube (2), corolla (3), and crest (4)]; C, complete flower; D, open flower showing disposition of sexual organs inside the corolla.

success data were collected in the three populations during the spring of 2004 and 2005. The remaining experiments were performed in the Colldecarrera population during the spring of 2005.

FLOWER MORPHOLOGY

Morphometric analyses were performed on 78 flowers from the Colldecarrera population by collecting the following measurements: angle of flower presentation, and length of wing-sepals, corolla, corolla tube, and keel appendage (Fig. 2B). Moreover, whenever nectar robbing occurred (following Inouye, 1980), robbery signs and ovary damage were recorded for every flower. The presence of nectar guidelines was studied under ultraviolet (UV) light in flowers of recent herbarium vouchers (deposited at the herbarium of the University of Aveiro, Portugal, AVE).

FLORAL LIFESPAN

The floral lifespan was studied by marking 23 flowers before opening. The flowers were then monitored daily until senescence. The time of pollen release and the relative position of the style and anther were recorded to evaluate whether delayed self-pollination occurred through style and stigma rotation.

PATTERNS OF NECTAR PRODUCTION

Daily nectar production was quantified in 37 flowers randomly selected from the Colldecarrera population.

The patterns of nectar production throughout the lifespan of the flower were evaluated by quantifying the nectar in 12 flowers for each age class (day –1, 0 or prior to anthesis, 1, 2, 3, 4, 5, 6, 7, and 8). In both treatments, the flowers were bagged 24 h before extracting the nectar. Nectar production was measured with a capillary micropipette, and the sugar concentration was determined (percentage by weight) with a portable refractometer. The amount of sugar produced by each flower was calculated according to Prys-Jones & Corbet (1987).

POLLEN AND OVULE PRODUCTION

In order to preview the reproductive system of this species, the P/O ratio was determined (Cruden, 1977). The number of pollen grains per flower was estimated in ten anthers of bud flowers from distinct individuals of each population, according to the following procedure: each anther was placed in a drop of glycerine 50% over a microscope slide, opened, and squashed beneath a cover slip; the pollen grains were counted under a light microscope (amplification, ×100). The number of ovules was evaluated in the same flowers as used for pollen counting. The number of aborted pollen grains was excluded from the P/O ratio determination.

STIGMA RECEPTIVITY

To determine the stigma receptivity throughout the lifespan of the flower, pollen germination on stigmas

and pollen tube growth along the style were assessed. Flowers were emasculated, bagged prior to anthesis, and marked with the day of flower opening. All flowers were hand-pollinated with a fresh pollen mixture collected from ten distinct individuals. The hand-pollinations were made in 10–12 flowers of each age class (day 0 or prior to anthesis, 1, 2, 3, 4, 5, 6, 7, and 8). All the flowers were collected 24 h after pollination and harvested in ethanol 70% for subsequent examination of pollen tube development. Pistils were cleared and softened with 8 M sodium hydroxide for 4 h, rinsed in distilled water, and stained overnight with aniline blue 0.05% prepared in 0.1 M potassium phosphate (Dafni, Kevan & Husband, 2005). Pistils were then placed on a microscope slide with a drop of glycerine 50% and squashed beneath a cover slip. Samples were observed using an epifluorescence microscope with a UV-2A filter cube. The number of pollen tubes growing throughout the stigmatic papillae and style was recorded. The percentage of receptive stigmas was calculated for each age class.

POLLEN GERMINATION

Pollen germination throughout the lifespan of the flower was assessed on stigmas. A set of recipient flowers was emasculated and bagged prior to anthesis. Another set of flowers was bagged and marked with the day of flower opening to function as pollen donors. Each recipient flower (in middle age) was hand-pollinated with pollen from a donor flower in a total of 12 pollinated flowers per age class (as defined above). All the receptive flowers were collected 24 h after pollination and harvested in ethanol 70%. Pollen grain germination was examined following the procedure described above.

HAND-POLLINATION EXPERIMENTS

To determine the reproductive system, the effect of insect exclusion and pollen source on the fruit production, seed set, seed to ovule ratio, and seed weight were investigated. The following treatments were applied: (1) spontaneous autogamy: flowers were bagged with a mosquito net to exclude insect interactions ($N = 35$ flowers); (2) obligated autogamy: flowers were bagged and pollinated with their own pollen ($N = 35$ flowers); (3) geitonogamy: emasculated flowers were bagged and pollinated with pollen of flowers from the same plant ($N = 35$ flowers); (4) xenogamy: emasculated flowers were bagged and pollinated with a fresh pollen mixture collected from ten distinct plants ($N = 35$ flowers); (5) supplementary pollination: flowers were pollinated with outcross pollen without bagging ($N = 30$ flowers); (6) control:

flowers without treatment ($N = 42$ flowers). Each flower belonged to a distinct plant. After 4–5 weeks, fruit and seed production was recorded and fruits and seeds were collected for seed weight measurement. Before weight measurement, seeds were dehydrated under natural conditions and maintained in a vacuum desiccator with silica gel for 24 h.

Moreover, the self-incompatibility system was investigated in order to determine at which level the inhibition of self-pollination occurs. All the treatments described above were repeated and, 24 h after pollination, flowers were collected, harvested in ethanol 70%, and pollen grain germination and pollen tube development were examined under an epifluorescence microscope (as described above).

INDICES RELATED TO THE BREEDING SYSTEM

Using the results obtained in the hand-pollination experiments, several indices related to the breeding system were calculated. In order to discriminate between the possible effects of dichogamy and genetic incompatibility, when necessary, self-pollination performance was determined using the results of geitonogamous pollen transfer.

Selfing rate and inbreeding depression: The selfing rate (S) was calculated according to Charlesworth & Charlesworth (1987):

$$S = (P_x - P_o)/(P_x - P_s)$$

where P_x is the chosen indicator of reproductive performance after cross-pollination, P_s after autogamy, and P_o under natural pollination. The seed to ovule ratio was chosen as the indicator of reproductive performance.

The level of inbreeding depression (δ) was determined by the relationship between the fruit set, seed to ovule ratio, and seed weight of geitonogamous-pollinated flowers (w_s) and cross-pollinated flowers (w_x) (Charlesworth & Charlesworth, 1987):

$$\delta = 1 - (w_s/w_x)$$

Self-compatibility and self-fertilization indices: The self-compatibility index (SCI) and the self-fertilization index (SFI) were calculated according to Lloyd & Schoen (1992). SCI is the ratio between the mean seed to ovule ratio for manually self-pollinated flowers and the mean seed to ovule ratio for cross-pollinated flowers. SFI is the ratio between the mean seed to ovule ratio for spontaneous self-pollination transfer and the mean seed to ovule ratio for cross-pollinated flowers.

Percentage of pollination limitation (PPL): A measure of the extent of reproductive success limitation by insufficient pollen delivery, or by resources, was calculated using the formula:

$$PPL = [100 \times (PS - C)]/PS$$

where *PS* is the seed to ovule ratio of pollen-supplemented plants and *C* is the seed to ovule ratio of control plants (Jules & Rathcke, 1999).

POLLEN LIMITATION AND FRUIT PRODUCTION

In order to evaluate whether the reproductive outcomes were influenced by pollen limitation throughout 2004 and 2005, the pistils of 80–100 flowers from all populations were observed, and the presence/absence of pollen grains over the stigmas and pollen tube development was analysed (as described above).

To evaluate the constancy of reproductive success, fruit and seed production was assessed in all populations. Randomly selected flowers were marked during the same flowering period, and fruit and seed production was recorded and the fruits and seeds were collected when mature.

STATISTICAL ANALYSIS

Descriptive statistics were calculated for flower traits, nectar production, P/O ratio, and seed weight, and are presented as the mean and standard deviation of the mean. Differences in nectar volume, sugar concentration, and sugar quantity throughout the lifespan of the flower, as well as differences in pollen tube number along the style as a function of the pollination treatment, were assessed using Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks, employing Dunn's method for pairwise multiple comparison. Differences between populations in pollen production, percentage of aborted pollen grains, and P/O index were analysed using one-way ANOVA followed by Tukey's test. A logarithmic transformation of the original data was applied to the number of aborted pollen grains. The effect of flower age on the ability of pollen to germinate and the effect of hand-pollination treatment on seed weight were analysed using one-way ANOVA followed by Tukey's test [general linear model (GLM) procedure because of unbalanced data].

The effect of hand-pollination treatment on the proportion of pollen tubes, fruit set, and seed to ovule ratio was analysed using a χ^2 test for the comparison of more than two proportions, followed by a multiple comparison test (Zar, 1984). The proportions of receptive stigmas throughout the lifespan of the flower, and the proportions of pollen loads and pollen tubes, fruit set, and seed to ovule ratio between populations and

years (categorical data approximated by a binomial distribution), were analysed using a logistic regression model with a link function (logit).

RESULTS

FLOWER MORPHOLOGY

Polygala vayredae flowers appear in small axillary inflorescences of one to three flowers in a minute peduncle (Fig. 2A). The flowers are large (16.2 ± 0.93 mm in length) and zygomorphic, with a remarkable superficial resemblance to Papilionaceae flowers (Fig. 2C). The calyx has five sepals, the basal two smaller, the upper one cup shaped, and the two laterals petaloid wings (wing-sepals), 15.9 ± 0.99 mm in length, apparently involved in visitor advertisement. The corolla has three petals partially fused, the upper two almost completely fused forming a long corolla tube (14.0 ± 0.72 mm in length) and the lower forming a bowl or big cup (keel), extremely noticeable because of its sulphur yellow colour, connected with the corolla tube by a fold and functioning as a hinge; this lower petal has a fringed crest (2.4 ± 0.46 mm wide) that provides a surface for insect foothold, and is functionally analogous to the keel of Papilionaceae, enclosing and protecting the reproductive organs (Fig. 2D). The filaments of the stamens are united into a tube slit which is connate to the base of the corolla tube; the filaments and the style run along the corolla tube and the eight anthers and stigma are enclosed in the keel. The ovary is superior, bilocular, with one pendulous ovule per locule; the stigmatic area is divided into two lobes, one anterior fertile, with wet stigmatic papillae, and another posterior sterile, with a basket shape specially adapted to pollen presentation (secondary pollen presentation, with concealed pollen presentation within the keel) (Fig. 2D). Flowers are exposed to potential visitors at an angle of 143°47′ ± 10°60′ with the petiole. No nectar guidelines were observed.

FLORAL LIFESPAN

The flowers are open for 8 ± 1.1 days and, during that time, the style curves slightly back and downwards, the anthers wilt, and the keel colour changes from light yellow to orange. Anthers open in bud and secondary pollen presentation begins (Fig. 2D). During secondary pollen relocation, the majority of the pollen grains are deposited on the style basket for presentation to the floral visitors. Afterwards, the downward movements of the keel help to recharge the basket style with small doses of the remaining pollen grains. Despite the movement of the style during maturation, stigmatic papillae and anthers do not come into contact. Nevertheless, occasional selfing or

self-interference cannot be totally excluded as pollen is secondarily presented near the stigmatic papillae.

PATTERNS OF NECTAR PRODUCTION

Nectar is produced at the base of the ovary in a conspicuous gland located between the two upper petals (Fig. 2D). The flowers produce 1.9 ± 0.86 μL of nectar with 16.0 ± 6.23% of sugar, and are able to continue nectar production after the removal of nectar.

In relation to flower age, nectar production starts before anthesis and precedes pollen dehiscence. Although highly variable (Fig. 3), a clear pattern can be observed, with a high nectar production in the first 3 days of anthesis and an accentuated decrease in both nectar volume ($H = 127.6$, $P < 0.001$; Fig. 3A) and sugar amount ($H = 161.3$, $P < 0.001$; Fig. 3C) afterwards. With regard to sugar concentration, larger values were registered on the day of anthesis ($H = 157.1$, $P < 0.001$), coinciding with pollen dehiscence (Fig. 3B).

The nectar accumulates at the base of the corolla tube and in the upper sepal. Long-tongued bumblebees (*Bombus pascuorum*) were observed visiting *P. vayredae* flowers (legitimate visits). Nectar and pollen robbery occurred and was clearly indicated by holes made by robbers in the upper sepal or corolla tube and keel, respectively. The observation of flowers by morphometric analysis revealed the frequent occurrence of nectar robbing (70.5%), mainly by *Bombus terrestris* (field observations) and, to a lesser extent, pollen robbing (8.9%) by Thysanoptera (frequently found within the robbed flowers). Despite the high frequency of nectar robbery, only in 1.9% and 2.4% of cases the ovary and nectar gland were damaged, respectively.

POLLEN AND OVULE PRODUCTION

Polygala vayredae flowers produce 5428 ± 1046.3 pollen grains per flower, a variable percentage of

which are aborted grains (0.8–56.7%). Each flower always produces two ovules. Excluding the aborted grains, flowers present a P/O ratio of 2346 ± 599.2 (Table 1).

Differences in total pollen production per flower and P/O ratio were detected between populations ($F = 3.532$, $P = 0.043$; $F = 8.443$, $P = 0.0001$, respectively), decreasing from Montmajor to Colldecarrera and Serrat dels Boixos populations. No differences were found in the number of aborted pollen grains ($F = 1.272$, $P = 0.296$) (Table 1).

STIGMA RECEPTIVITY AND POLLEN GERMINATION

The observation of flower maturation seems to indicate a protandrous flower, as the pollen is released into the style basket before anthesis. Nevertheless, the study of pollen viability revealed that pollen is able to germinate during the entire lifespan of the flower, only decreasing slightly in the final days ($F = 5.760$, $P < 0.001$; Fig. 4). By contrast, the stigma receptivity varies with flower age: it increases significantly from day 0 to day 3 (Wald $\chi^2 = 10.2$, $P = 0.017$), when it reaches a maximum, and then decreases slightly, although always maintaining a large percentage of receptive flowers until senescence (Wald $\chi^2 = 3.5$, $P = 0.627$; 61.5% of flowers were receptive in the final days; Fig. 4).
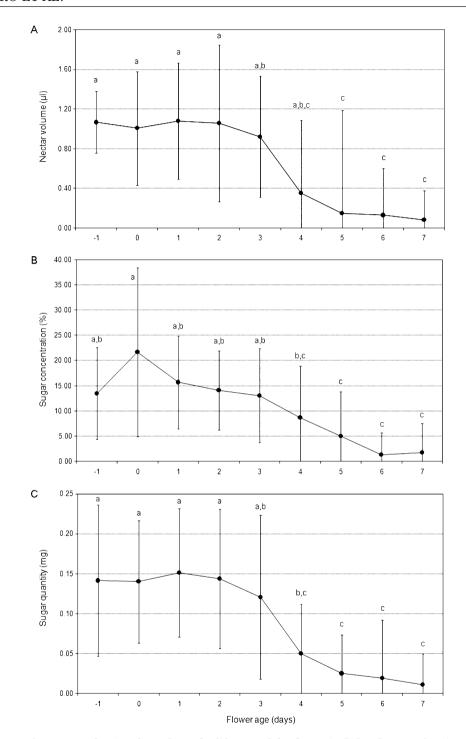
HAND-POLLINATION EXPERIMENTS

The results of pollinator exclusion and hand-pollination experiments are presented in Table 2. The pollen source affected significantly the fruit set and seed to ovule ratio ($\chi^2 = 110.6$ and 137.6, $P < 0.001$ respectively). No fruit production was observed after spontaneous autogamy, revealing that *P. vayredae* flowers are incapable of self-fertilization (SFI = 0), and thus depend on pollen transfer vectors to effect pollination. The fruit set and seed to ovule ratio in autogamous (14.3% and 7.1%, respectively) and gei-

Table 1. Total pollen, aborted pollen grains, and pollen to ovule ratio (P/O) produced per flower in three populations of *Polygala vayredae*

Population	Total pollen per flower	Aborted pollen per flower	P/O ratio*
1 – Montmajor	6080 ± 1103.3a	359 ± 191.8a	2860 ± 541.8a
2 – Serrat dels Boixos	5203 ± 875.2a,b	927 ± 1064.9a	2138 ± 507.8b
3 – Colldecarrera	5001 ± 901.4b	917 ± 929.7a	2042 ± 400.5b
Total	5428 ± 1046.3	735 ± 839.4	2346 ± 599.2

The values are given as the mean and standard deviation of the mean. Differences between populations were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's test. Different letters reveal statistical differences at $P < 0.05$.
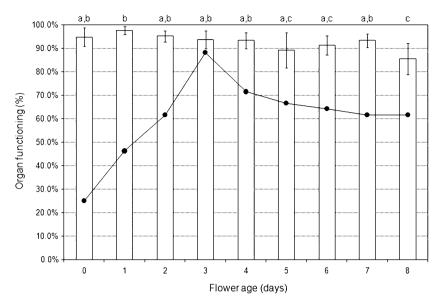
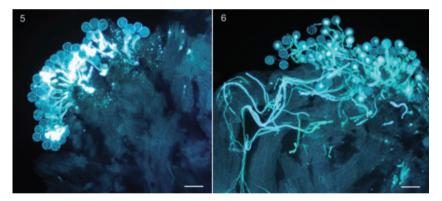*Pollen to ovule ratio was calculated excluding the aborted pollen grains.

Figure 3. Patterns of nectar production throughout the lifespan of the flower in *Polygala vayredae*: A, nectar volume (μl); B, sugar concentration (%); C, sugar quantity (mg) (all according to flower age). Values are given as the mean and standard deviation of the mean. Different letters reveal statistical differences at *P* < 0.05.

tonogamous (8.6% and 4.3%, respectively) pollinations were low. In addition, the autogamous and geitonogamous treatments produced similar fruit set and seed to ovule ratios, indicating the lack of a dichogamous system that prevented mediated self-

fertilization. This is in agreement with the results obtained for male and female functioning (Fig. 4). Xenogamous and supplementary pollination treatments produced a higher fruit set (82.9% and 93.3%, respectively) and seed to ovule ratio (51.4% and

Figure 4. Stigma receptivity (filled circles), given as the percentage of flowers that enabled pollen tube growth along the style, and pollen germination (open bars), given as the percentage of germinated pollen grains over stigmas, according to flower age in *Polygala vayredae*. Different letters reveal statistical differences at $P < 0.05$ in pollen germination.

Figures 5–6. Pollen tube development at the stigmatic papillae and beginning of the style in *Polygala vayredae* after hand-pollination experiments. Fig. 5. Geitonogamous pollination. Fig. 6. Xenogamous pollination. Scale bars, 100 μm.

66.7%, respectively). As a result, the estimated SCI (0.084) and the selfing rate ($S = 0.308$) were low, indicating the presence of an auto-incompatibility system. Inbreeding depression, based on the outcomes of selfing and outcrossing treatments, was high for fruit set ($\delta = 0.896$) and seed to ovule ratio ($\delta = 0.916$), but relatively low for seed weight ($\delta = 0.096$). Under natural conditions, the fruit set (47.6%) and seed to ovule ratio (36.9%) were significantly lower than in the supplementary pollination treatment, indicating the occurrence of pollen limitation in the Colldecarrera population during the spring of 2005. Moreover, this was supported by a relatively high PPL (44.7%).

With regard to seed weight, differences were observed among pollination treatments ($F = 6.19$, $P < 0.001$). Supplementary pollination produced sig-

nificantly heavier seeds than did self-pollination (autogamy and geitonogamy) and control pollination ($P < 0.01$). Autogamous and geitonogamous pollinations produced lower seed weights (Table 2).

The observation of pollen tube development as a function of the pollen source revealed that, in autogamous and geitonogamous pollinations, the pollen grains were able to germinate, but in most cases failed to pass the stigmatic papillae (Table 2, Fig. 5). By contrast, in xenogamous pollinations, pollen tube development along the style was observed in almost all individuals (91.4%) and in higher numbers (5.0 ± 3.03 pollen tubes; Fig. 6). The numbers of pollen tubes in supplementary and control pollinations were slightly higher than in xenogamous pollinations, probably as a result of the

Table 2. Number of pollen tubes, fruit and seed production, and seed weight for *Polygala vayredae* flowers under different hand-pollination treatments

Hand-pollination treatment	N	Number of pollen tubes*	Number of fruit (fruit set)	Number of seeds (seed to ovule ratio)	Seed weight (mg)†
Spontaneous autogamy	35	0a (0%a)	0 (0%)a	0 (0%)a	–
Obligatory autogamy	35	0.4 ± 0.82a (17.1%b)	5 (14.3%)b	5 (7.1%)b	7.89 ± 0.942a
Geitonogamy	35	0.5 ± 0.96a (22.9%b)	3 (8.6%)b	3 (4.3%)b	8.73 ± 1.251a,b
Xenogamy	35	5.0 ± 3.03b (91.4%c)	29 (82.9%)c	36 (51.4%)c	8.73 ± 1.251a,b
Supplementary pollination	30	10.4 ± 6.16b (93.3%c)	28 (93.3%)d	40 (66.7%)d	9.43 ± 1.728b
Control	42	8.9 ± 7.22b (78.6%d)	20 (47.6%)e	31 (36.9%)e	8.17 ± 1.560a
ANOVA		H = 150.5‡			F = 6.19‡
χ² comparison for proportions		120.7‡	110.6‡	137.6‡	

The number of pollen tubes and seed weights (mg) are given as the mean and standard deviation of the mean. The effect of pollination treatment on the proportion of flowers with pollen tubes, fruit set, and seed to ovule ratio was analysed using a χ² test for the comparison of more than two proportions, followed by a multiple comparison test (Zar, 1984). Differences in the number of pollen tubes per flower between treatments were analysed using a Kruskal–Wallis one-way analysis of variance (ANOVA) followed by Dunn's method. Differences in seed weight between treatments were analysed with a one-way ANOVA followed by Tukey's honestly significant difference (HSD) test [general linear model (GLM) procedure]. Different letters reveal significant differences at *P* < 0.01.
*The percentage of individuals with pollen tube development along the style is given in parentheses.
†Because of the reduced number of seeds, the mean seed weight of obligatory autogamy and geitonogamy was jointly assessed.
‡*P* < 0.001.

higher quality of the pollen grains delivered by pollination vectors.

POLLEN LIMITATION AND FRUIT PRODUCTION

The pollen loads on stigma, pollen tube development, and fruit and seed production are given in Figure 7. All parameters related to possible pollen limitation varied significantly between populations and years (Wald χ² > 37.0, *P* < 0.0001). Overall, the values of all parameters were lower in all populations during 2004. Moreover, the values for all analysed parameters decreased from the Colldecarrera to Montmajor population during 2005.

With regard to the presence of pollen on the stigmas, the results showed that, in general, the pollinator activity in this plant was low. This was evident during 2004 in all populations (Fig. 7A) and during 2005 in the Montmajor population (Fig. 7B), where only 28.6–36.6% of the flowers received visit(s). In addition, although during 2004 the estimated visitation rates were similar, during 2005 the pollinator activity varied significantly between populations (Fig. 7).

Secondarily, the quantity and quality of the delivered pollen also affected the reproductive outcome of *P. vayredae*. This was clearly evident in the Montmajor population during 2004 where, in flowers that received visit(s), pollen tube development along the style was observed in only 21.4% of cases (i.e. a decrease from 28.6% of visited flowers to only 6.1% where pollen tube development was observed; Fig. 7A), and in all populations during 2005 (Fig. 7B).

Finally, a significant decrease from the number of flowers presenting developed pollen tubes to the number of flowers that set fruit was also observed, mainly during 2005 (for example, in the Serrat dels Boixos population, a decrease from 52.6% of flowers with pollen tube development to only 28.9% where fruit production was observed; Fig. 7B), although some differences were also recorded during 2004.

DISCUSSION

The flower is the structure directly involved in pollinator attraction, and its morphological and functional traits affect the reproductive success of the plant. Thus, floral features may have great importance in narrow endemic species (Navarro & Guitián, 2002). The floral morphology of *P. vayredae* is especially adapted for insect attraction. This species presents large zygomorphic flowers, with the androecia and gynoecia concealed in the keel, advertisement wings and long corolla tubes, bright colours, and a platform for visitors to land and gain a foothold (Fig. 2). Furthermore, associated with this elaborate
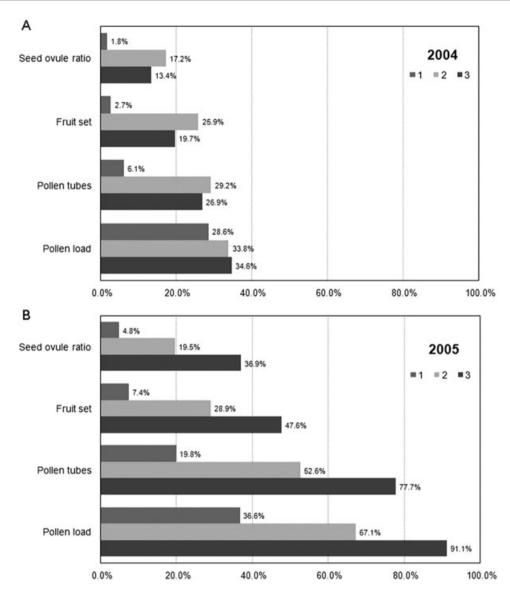
Figure 7. Pollen loads on stigma, pollen tube development, fruit set, and seed to ovule ratio of *Polygala vayredae* in three populations (1, Montmajor; 2, Serrat dels Boixos; 3, Colldecarrera) in 2004 (A) and 2005 (B).

arrangement of floral pieces, several functional traits reveal an adaptation for entomophily. Flowers have a large lifespan, which enhances the probability for receiving a visit (Ashman & Schoen, 1996), produce abundant nectar-like rewards for floral visitors, and present a specialized mechanism of secondary pollen presentation.

Ashman & Schoen (1994, 1996) predicted optimal floral longevity as a trade-off between resource allocation to floral construction and floral maintenance. Assuming a fixed availability of resources, optimal floral longevity is determined by the interaction between the daily cost of flower maintenance in relation to the cost of producing a new flower (floral maintenance cost) and the rates of male and female

fitness accrual over time (Ashman & Schoen, 1994, 1996). According to this, long-lived flowers are selected when fitness accrual rates and floral maintenance costs are low, whereas short-lived flowers are selected when fitness accrual rates and floral maintenance costs are high. Furthermore, the model assumes that plants adapt to low levels of pollinator activity through the evolution of differences in floral longevity. In a group of 11 species, Ashman & Schoen (1994) estimated a daily cost of flower maintenance ranging from 2% to 9% of the cost of producing a new flower and variable male and female fitness accrued. Despite the lack of data on transpiration, respiration, and nectar production, which constitute important energetic costs (for example, Southwick, 1984; Pyke,

1991), these results support the floral longevity model. *Polygala vayredae* balances the production of few flowers per ramet by presenting large and long-lived flowers. The dependence on pollinators for seed production and the very low visitation rates (see 'Results' section) seem to be the factors that explain the long lifespan of *P. vayredae* flowers.

Floral rewards play an important role in most flower–visitor interactions. Flowers have evolved many rewards to attract pollinators, frequently as nectar or pollen, in exchange for transporting their male gametes (Dafni *et al*., 2005), and *P. vayredae* is no exception. In *P. vayredae* flowers, although nectar is offered as a reward, pollen is protected within the corolla and presented in an accurate mechanism of pollen transfer. Nectar production and pollen release occur simultaneously at flower anthesis, when the higher nectar concentration is offered. Moreover, nectar composition also affects visitor behaviour and can generate different patterns of pollen export and reception (Thomson, 1986; Harder & Thomson, 1989). *Polygala vayredae* flowers produce abundant nectar with an unexpectedly low concentration of sugar for bumblebee-pollinated flowers (for example, Corbet, 1978; Chalcoff, Aizen & Galetto, 2006; but see Percival, 1965). In addition, several authors have demonstrated that flowers invest more in the export of pollen through offering rewards than in pollen reception (Thomson, 1986; Harder & Thomson, 1989). Pleasants (1983) argued that variability in nectar production throughout the lifespan of the flower is a way of offering different reward assemblages for flower visitors. Nectar production in *P. vayredae* flowers is higher during the male phase and decreases when the female phase begins (see Figs. 3 and 4) in a strategy to improve male function through pollen export (Mitchell, 1993). Nevertheless, after the decrease in nectar production, as stigmas maintain a high level of receptivity and pollen grains sustain their ability to germinate, cross-pollination may still occur with the other floral traits involved in pollinator attraction.

Another feature that cannot be forgotten is nectar robbing. In *P. vayredae*, the fact that nectar is concealed at the base of a long corolla tube leads to frequent nectar robbery by visitors that are incapable of reaching this reward in other ways (for example, *Bombus terrestris* proboscides measure 5.8 ± 0.34 mm, but the *P. vayredae* corolla tube measures 14.5 ± 0.78 mm). This robbing attitude may not affect visitation by actual pollinators (*Bombus pascuorum*), as flowers are able to produce more nectar after removal, and a high robbing frequency can co-occur with high visitation rates (Navarro, 2001). Nevertheless, further studies are needed to understand the impact of nectar robbery on nectar production patterns and visitation rates, and the

consequence of continuous resource removal on fruit production and seed weight.

The efficiency of the insect visit can be improved by the mechanism of secondary pollen presentation. This mechanism evolved to spatially join the male and female functions through pollen relocation in a specialized area of the gynoecium. It has been described as a strategy to improve accuracy in pollen removal and deposition, which will result in the enhancement of male and female fitness (Howell, Slater & Knox, 1993; Yeo, 1993; Ladd, 1994). Different mechanisms may have evolved in parallel with secondary pollen presentation to reduce self-interference and self-fertilization, such as dichogamy and self-incompatibility systems (Lloyd & Webb, 1986; Howell *et al*., 1993). Self-interference and self-fertilization have already been discussed by Brantjes (1982) in *P. monticola* var. *brizoides* and *P. vauthieri*. This author reinforced the importance of the quality of the first visit in precise pollen removal and deposition, such that, when inefficiently performed, auto-pollination (if a mechanism to prevent selfing is absent) or clogging of the stigmatic papillae with its own pollen is prone to occur. However, parallel to secondary pollen presentation, mechanisms of delayed self-pollination could have evolved. Delayed selfing allows outcrossing when pollinators are present and provides reproductive assurance when pollinators are scarce or unpredictable (Seed, Vaughton & Ramsey, 2006). This mechanism seems to occur within Campanulaceae and Asteraceae by means of stigmatic lobe curvature (Faegri & van der Pijl, 1979; Cerana, 2004), and in several species of Polygalaceae by means of enclosed fertile and sterile stigmatic lobes (for example, *P. rugellii*, *P. nana*, and *P. sectacea*; Weekley & Brothers, 2006).

The results of the hand-pollination experiments showed that *P. vayredae* requires pollinator visits in order to produce fruit, as no fruit was set after pollinator exclusion (Table 2). Despite the occurrence of secondary pollen presentation near the receptive papillae, and contrary to what is observed in other species of the genus, *P. vayredae* does not possess a mechanism of self-fertilization or delayed self-fertilization that provides reproductive assurance when visitors are scarce or inefficient. In addition, autogamy and geitonogamy resulted in a very low fruit and seed production, revealing the presence of an auto-incompatibility system, with the stigmatic surface being the site of pollen grain inhibition for self-pollinated flowers. The occurrence of an auto-incompatibility system will promote outcrossing and prevent inbreeding depression (Silva & Goring, 2001). A self-incompatibility system has also been described for *P. arillata*, where a fruit set of 2.3% was obtained after autogamy (Devy & Davidar, 2006). This also contrasts with general assumptions made for several

Polygala species, where autogamy regularly occurs (Venkatesh, 1955, 1956; Dube, 1962; Miller, 1971; Brantjes, 1982; Lack & Kay, 1987; Norderhaug, 1995; Weekley & Brothers, 2006), although most of these were based only on flower morphology, rather than considering the functional aspects. In *P. vayredae*, despite the presence of an incompatibility system that prevents auto-pollination, the degree of self-interference as a result of male and female proximity is unknown. The reproductive system of *P. vayredae* is in accordance with the estimated P/O value, which suggests a reproductive system between facultative and exclusive xenogamous (Cruden, 1977).

Pollen availability and quality are two principal determinants of female reproductive success (Haig & Westoby, 1988; Griffin & Barrett, 2002), and pollen limitation has been shown to be widespread, especially in animal-pollinated species (Burd, 1994; Larson & Barrett, 2000; Ashman *et al*., 2004). Furthermore, the available resources and resource allocation also play a major role in the final female reproductive success (Wesselingh, 2007). When *P. vayredae* flowers were supplemented with outcross pollen, a significantly higher fruit set was observed in comparison with the control, revealing pollen limitation in the Colldecarrera population during the spring of 2005. Furthermore, the scarcity of pollen over the stigmas from three populations over the 2 years indicates that pollen limitation appears to be frequent in this species, as the visitation rate is low despite the attractive floral traits. During 2004, pollinator activity seemed to be the major factor affecting fruit production in the Colldecarrera and Serrat dels Boixos populations, whereas, in Montmajor, in addition to this factor the quantity and/or quality of pollen also played an important role for the final reproductive outcome. This lower female fitness may result from the population size, as it has been shown that a small population size can reduce the number of compatible mates, especially in species with self-incompatibility barriers, and increase the relatedness amongst possible mates (Ågren, 1996; Ward & Johnson, 2005). This, associated with resource limitation, may have affected the fruit and seed production in the small population of Montmajor. Furthermore, co-flowering plants may result in pollinator competition, increased heterospecific pollen delivery, and/or stigma clogging by heterospecific pollen (Campbell, 1985; Gross, 1996). In the Montmajor population, there are other species that begin to flower at the peak of the *P. vayredae* flowering period, competing for the potential pollinators of this species, and diminishing the visitation rate of *P. vayredae* (S. Castro, unpubl. data). In the Serrat dels Boixos and Colldecarrera populations, *P. vayredae* is the main species flowering. During 2005, the pollinator activity was higher than in 2004, and significant differences were observed between the populations. This resulted in a higher fruit and seed production in all populations. Despite this, several other features, such as resource limitation and pollination quality, affected the reproductive outcome, leading to a significant reduction in fruit and seed production. Pollination quality (Herrera, 1987) is determined by pollinator behaviour, which can be influenced by plant density (for example, Guitián, Guitián & Navarro, 1993; Grindeland, Sletvold & Ims, 2005). Colldecarrera and Serrat dels Boixos are large populations with dense clusters of *P. vayredae* and reduced seed dispersal (S. Castro, unpubl. data); flower visitors, when exploiting several flowers of these dense clusters, promote geitonogamous pollination or pollination between closely related individuals, and reduce female fitness. Nevertheless, the impact of pollen limitation on the population dynamics of *P. vayredae* is still unknown. Furthermore, *P. vayredae* can circumvent this limitation by being a perennial plant.

This study has shown that the floral traits of *P. vayredae* are indicative of a xenogamous and entomophilous flower, in accordance with the breeding system of this species. The low fruit production in *P. vayredae* is largely a result of scarce or unreliable pollinators and poor pollen quality. In addition, the available resources may be a limiting factor affecting final fruit production, mainly in the more arid/adverse populations. The reproductive strategy of *P. vayredae* prevents inbreeding depression by a self-incompatibility system, and, in years of scarce pollinators, it is overcome by the plant habit. Further studies on pollinator communities, efficiency and reliability, nectar biology, seed dispersal, and genetic structure are being developed to further understanding the biology of this species.

ACKNOWLEDGEMENTS

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