



**Sílvia Raquel Cardoso  
Castro**

**BIOLOGIA REPRODUTIVA E CONSERVAÇÃO DO  
ENDEMISMO *POLYGALA VAYREDAE***

**REPRODUCTIVE BIOLOGY AND CONSERVATION OF  
THE ENDEMIC *POLYGALA VAYREDAE***





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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Paulo Cardoso da Silveira, Professor Auxiliar Convidado do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Doutor Luis Navarro Etxeberria, Professor Associado do Departamento de Biologia Vegetal e Ciências do Solo da Faculdade de Biologia da Universidade de Vigo.

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## palavras-chave

apresentação secundária de pólen, conservação, dispersão de sementes, êxito reprodutivo, interacções planta-animal, interacções pólen-pistilo, limitação de pólen, morfologia floral, néctar, polinização, selecção de rasgos florais, sistema de reprodução.

## resumo

Existe um consenso generalizado de que o estudo da biologia reprodutiva de espécies endémicas, raras ou ameaçadas é essencial para compreender o seu estado actual e no desenvolvimento de estratégias adequadas de conservação *in situ* e *ex situ*. *Polygala vayredae* Costa (Polygalaceae) é uma espécie endémica da cadeia pré-Pirenaica oriental classificada como vulnerável de acordo com as categorias da IUCN. Os objectivos da presente Tese de Doutoramento consistiram em estudar a biologia reprodutiva de *P. vayredae*, avaliando de que forma pode afectar o ciclo de vida, em fornecer informação de base para futuros programas de conservação e em explorar em maior profundidade as interacções planta-animal e os processos de evolução de determinados traços florais. Para alcançar estes objectivos foram investigados os seguintes tópicos: biologia floral e sistema de reprodução (Capítulo 2), mecanismo de apresentação secundária de pólen (ASP, Capítulo 3), ecologia da polinização (Capítulo 4) e mecanismos de dispersão (Capítulo 5). Os resultados revelaram que (1) *P. vayredae* depende estritamente de vectores de polinização para produzir sementes, (2) as suas características florais (corola fechada, accionada por comportamentos específicos do insecto) limitam o conjunto de polinizadores eficientes, (3) a sua longevidade floral favorece a transferência de pólen mas apresenta também custos reprodutivos sobre o êxito feminino, (4) a ASP acarreta custos reprodutivos sobre o êxito das componentes masculina e feminina, (5) as flores de *P. vayredae* são visitadas por um diversificado conjunto de insectos, dos quais apenas quatro espécies se comportam como polinizadores eficientes, (6) os polinizadores eficientes são, frequentemente, escassos e variáveis ao longo do tempo e do espaço, (7) os visitantes ineficientes exercem um efeito negativo sobre o comportamentos dos polinizadores eficientes e consequentemente sobre o êxito masculino (reduzindo o fluxo de pólen) e feminino (reduzindo a produção de frutos e sementes), (8) a dispersão dos frutos alados a longas distâncias encontra-se largamente reduzida, e finalmente, (9) a dispersão é maioritariamente efectuada por formigas que dispersam as sementes a distancias reduzidas, com o conjunto de espécies variando grandemente entre populações e anos. Perante estas observações podemos concluir que *P. vayredae* apresenta os seguintes problemas reprodutivos: limitação de pólen/polinizadores e curtas distâncias de dispersão. Os programas futuros de conservação que considerem estes pontos, conservando a fauna da área e protegendo as interacções planta-animal, promoverão a manutenção das populações e, consequentemente, a conservação desta espécie.



## keywords

conservation, floral morphology, floral traits selection, nectar, plant-animal interactions, plant fitness, pollen limitation, pollen-pistil interactions, pollination, reproductive system, secondary pollen presentation, seed dispersal.

## abstract

There is a consensus that studies on the reproductive biology of endangered, rare or threatened species are useful for understanding their current status and for evaluating *in situ* and *ex situ* management strategies. *Polygala vayredae* Costa (Polygalaceae) is a narrow endemic species from oriental pre-Pyrenees classified as vulnerable according with the IUCN categories. The aims of this PhD thesis were to study the reproductive biology of *P. vayredae*, to evaluate the implications of its reproductive features on its life cycle, to provide valuable background information for future management programs and deeply explore plant-animal interactions and the evolution of certain floral traits. To achieve the proposed objectives the following points were investigated: the floral biology and breeding system (Chapter 2), the singular secondary pollen presentation (SPP) mechanism (Chapter 3), the pollination ecology (Chapter 4) and the dispersal mechanisms (Chapter 5). The results revealed that (1) *P. vayredae* strictly depends on pollen vectors to produce seeds, (2) its floral features (closed corolla and the need to be triggered by specific behaviours of the insects) limit the spectrum of efficient pollinators, (3) its floral longevity favours the opportunity to pollen transfer but also have reproductive costs over female fitness, (4) SPP imposes several reproductive costs over male and female fitness, (5) a large spectrum of floral visitors were observed, but with only four species being efficient pollinators, (6) efficient pollinators were scarce and highly variable both along time and space, (7) inefficient visitors add a negative effect on the behaviour of efficient pollinators and on male (reducing pollen flow) and female components (reducing fruit set and seed ovule ratios), (8) long dispersal of the alate fruits is largely reduced, and finally, (9) dispersal is mainly performed by ants who are able to disperse seeds for short distances, with the spectrum of ant species being spatially and temporally variable. The general conclusions on the status of *P. vayredae* indicate that this species bears the following reproductive problems: pollen/pollinator limitation and short distance dispersal of the diaspores. Future management programs that account with this issues conserving the fauna of the area and protecting plant-animal interactions will facilitate the maintenance of the populations and thus its conservation.



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## **Abbreviations:**

AVE – Herbarium of University of Aveiro

CV – coefficient of variation

GLM – general linear models

GLZ – generalized linear/nonlinear models

GMT – Greenwich mean time

IUCN – International Union for Conservation of Nature and Natural Resources

PAS – periodic acid-Schiff

PCA – principal component analysis

PIPES – piperazine-N,N'-bis-2-ethanesulfonic acid

P/O – pollen ovule ratio

PP – pollen presenter

PPL – percentage of pollination limitation

S – selfing rate

SCI – self-compatibility index

SD – standard deviation of the mean

SE – standard error of the mean

SEM – scanning electron microscopy

SFI – self-compatibility index

SPP – secondary pollen presentation

UTM - universal transverse mercator

UV – ultraviolet

WWF – World Wide Fund for Nature





*"Hmmm! What pollinates you?"*

Illustration by Dr. HJ Svart (Adapted from *Practical pollination biology*, Dafni et al. 2005)



# Chapter 1

## Introduction





## Introduction

Natural habitats are currently being degraded at a rate that is unprecedented in history, and massive extinctions of biota are likely to follow as a result (Sala *et al.* 2000; Novacek and Cleland 2001). Under the pressures of globalization, continuous growth of human population, resources exploitation, and climate changes, the perturbation, displacement and/or destruction of natural habitats are real facts (Pimm *et al.* 1995; Novacek and Cleland 2001; Warren *et al.* 2001; Brook *et al.* 2003). Human activity has already transformed 40-50% of the ice-free land surface, using one-third of the terrestrial net primary productivity and causing the extinction of 5-20% of the species in many groups of organisms (Brook *et al.* 2003; Chapin 2003). Actually, the current rates of extinction are estimated to be 100 to 1,000 greater than the pre-human rates, as estimated through the fossil records (Pimm *et al.* 1995), with about 25% of the 250,000 estimated species of vascular plants potentially becoming extinct within the next 50 years (Raven 1987). Nonetheless, the loss of species is only a part of the ongoing biotic depletion. A decline of biodiversity within species as well as subspecies, races and populations is also occurring through dramatic reductions in their sizes (Myers 1989). Furthermore, as organisms are pieces of the ecosystems, their effects will cascade through a broad range of processes inherent to the tightly linked carbon, nutrient and water cycles (Tateno and Chapin 1997). Thus, the conservation of natural spaces and all the organisms therein assume a special and urgent importance in our days (Novacek and Cleland 2001). In this context, narrow endemic species assume particular interest as they could be especially sensible to habitat disturbance and may more easily diminish and disappear. Furthermore, they constitute interesting study models to evaluate the consequences of reduced population dimensions or population fragmentation.

There is a general consensus that studies on the reproductive biology of endangered, rare or threatened species may be useful for understanding some of the mechanisms involved in their actual status. Furthermore, several international organizations (*e.g.*, International Union for Conservation of Nature and Natural Resources, IUCN) have stressed the need to develop studies that supply basic information on the biology of endemic plants. The information obtained from such studies may be particularly useful for evaluating alternative *in situ* and *ex situ* management strategies for plant conservation. Despite of the abundant and increasing data on the taxonomy and chorology of plants, and particularly on endemic species, the information on its reproductive biology is still very limited, revealing the need for the development of studies on this important topic.

The ecological attributes of the plants and their interactions with the surrounding environment will affect several vital steps of its life cycle, being directly involved with population dynamics. The complex web of interactions involves intra- and inter-specific competition, predators and/or mutualists (e.g., Herrera 1993; Aizen *et al.* 2002; Strauss and Irwin 2004; Bronstein *et al.* 2006). Pollination is the first stage of sexual reproduction in flowering plants, with up to 90% of the species relying on animal vectors to disperse their pollen and achieve pollination. The outputs of pollination are expressed in number and quality of seeds, which are essential incomes for population growth. Furthermore, through pollen dispersal, gene flow along or among populations is also involved in this process, playing its role in the genetic composition of the populations (Rozzi *et al.* 1997). The disruption in plant-pollinator interactions, as a result of habitat fragmentation or species introduction, will thus affect on different ways all the intervenient species (Kearns *et al.* 1998; Ashworth *et al.* 2004; Harris and Johnson 2004). Dispersal is the second crucial step in the life cycle of a plant, determining the ability to spread from the mother plant, to reach new places, to achieve safer or favourable places for germination, and ultimately shaping the genetic structure of the populations (van der Pijl 1982). Therefore, understanding the processes of pollination and dispersal will provide relevant information not only in species conservation, but also on the processes of species evolution (Schemske *et al.* 1994).

## **Study system**

The Polygalaceae family is a natural group well characterised by the morphology of its flowers (papilionate flowers) and pollen grains (polizonocolporate pollen). The number of genera recognized within the family varies according with circumscriptions adopted by different authors. The most recent treatment recognize 22 genera and 800 to 1,000 species arranged in four tribes (Polygaleae, Moutabeae, Carpolobieae, and Xanthophylleae; Eriksen and Persson 2007). *Polygala* L. is the most representative genus of the family, comprising about 725 heliophyte species distributed around the world. It presents a remarkable pattern of regional endemisms and a significant representation in diversification centres as defined by the IUCN and World Wide Fund for Nature (WWF) programs (Paiva 1998). Furthermore, the high proportion of endemic species could suggest the occurrence of both neoendemisms and paleoendemisms (Paiva 1998; Forest *et al.* 2007). Nevertheless, despite the conservation importance of this genus and its diversified floral and diaspore morphology, few detailed studies have been performed so far, with the available information on its reproductive ecology being scarce.

*Polygala vayredae* Costa [Subgenus *Chamaebuxus* (DC.) Schb.] is an endemic species from oriental pre-Pyrenees, with a narrow distribution area of approximately 12 km<sup>2</sup> (Alta Garrotxa, Catalunya, Spain). It has been described as a relic that survived through the Quaternary glaciations in the restricted habitat pockets where it presently occurs. Until now, *P. vayredae* has received attention mainly from the botanist Antoni de Bolós (Bolós and Vayreda 1922; Bolós 1927; Bolós 1946). Nonetheless, this author focused on the description of its natural habitat, phytosociological associations and limits of distribution, with the reproductive biology of this species remaining largely neglected. Due to the scarce information on its biology and restricted distribution area, this species was considered important for conservation and therefore classified as a vulnerable species (*Lista Roja de Flora Vascular Española*, VV.AA. 2000). The preliminary observations revealed that *P. vayredae* appears in a very restricted area with several scattered populations where the plant occurs in high abundance. Its flowers are apparently highly specialized, similarly to what was already described for the related species *P. chamaebuxus* L. (Faegri and van der Pijl 1979). Also, populations of *P. vayredae* in different habitats seemed to present different reproductive outcomes, and its dispersal syndromes pointed for a long distance dispersal of the fruits. These observations revealed the need for further understanding the biology of this protected species.

## Objectives

The aims of this PhD thesis were (1) to study the reproductive biology of the narrow endemic species *Polygala vayredae*, (2) to evaluate the implications of its reproductive features on the life cycle, and finally, (3) to provide valuable background information for management programs under development in the natural area where this species occurs. Furthermore, the present work also aims to deeply explore plant-animal interactions and the evolution of certain floral traits of this species. The study presented in this thesis was developed in the three most representative populations of *P. vayredae* (Fig. 1.1).



**Fig. 1.1** Studied populations of *Polygala vayredae*: (A) Montmajor, (B) Colldecarrera and (C) Serrat dels Boixos (Alta Garrotxa, Catalunya, Spain).

To achieve the proposed objectives the following points were investigated:

#### *Floral biology and breeding system*

The flower is the structure involved in the formation of male and female gametes, fecundation and offspring production; thus, its functioning will determine the plant reproductive success. To understand flower functioning, flower morphology, male and female maturation and reproductive strategies were investigated in Chapter 2.1. Secondly, pollen tube development and style anatomy were studied in detail to understand the post-pollination processes (Chapter 2.2). Finally, the effect of flower longevity in reproductive success was also investigated (Chapter 2.3).

#### *Secondary pollen presentation*

*Polygala vayredae* presents an elaborated mechanism of pollen transfer involving the relocation of pollen near the stigmatic papillae. This adaptation is hypothesised as a mechanism for accurately deliver and receive pollen, increasing male and female fitness

and thus, directly influencing the reproductive outcome of the plant. However, the studies that experimentally evaluated the secondary pollen presentation mechanism are scarce. Thus, in Chapter 3.1 the consequences of such a mechanism in plant fitness are investigated for the first time, using *P. vayredae* as a case study.

#### *Pollination ecology*

The flower is the structure involved in visitor's attraction. As *P. vayredae* strictly depends on pollination vectors to produce seeds (Chapter 2.1), the effects of several floral features and nectar production on visitor's behaviour were evaluated in Chapter 4.1. Furthermore, the spatial and temporal variation of floral visitors and offspring production was assessed by monitoring three populations during three consecutive years (Chapter 4.2). Finally, as nectar robbing revealed to be a major feature in all populations, the patterns of nectar production and the effect of nectar robbing on both pollen flow and seed production were also evaluated (Chapter 4.3).

#### *Dispersal mechanisms*

The dispersal ability is a key factor determining plants distribution. *P. vayredae* presents two types of diaspores linked with two distinctive dispersal syndromes: alate fruits, apparently prepared for wind dispersal (anemochory), and seeds with an elaiosome specially involved in ant's attraction (myrmecochory). In Chapter 5.1, the mechanisms of primary and secondary dispersal were evaluated in three populations during three consecutive years, monitoring the spectrum of dispersal agents, seed predation and the effect of elaiosome removal on seed dispersal. Furthermore, the effect of elaiosome removal on *ex situ* seed germination was also evaluated.

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## Chapter 2

### Floral biology and breeding system

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

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## Abstract

*Polygala vayredae* is a narrow endemic species from oriental pre-Pyrenees. Despite of its conservation status and rarity, no information is available on its reproductive biology. As the flower is the structure directly involved with pollinator attraction, its morphological and functional traits have major effects on the reproductive success of the plant. In this work we studied the flower biology and breeding system of *P. vayredae* to evaluate how they affect the reproductive outcome in natural populations. Flower morphology, flower rewards and male and female functioning along flower lifespan 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 along two years, by observing the presence of pollen on the stigma, pollen tube development and fruit production. *P. vayredae* flowers are elaborated, 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 fruits. Low fruit production was observed in the studied populations, which was largely due to scarce, unreliable and/or inefficient pollinators and to the pollen quality. Also, available resources can 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.

**Keywords** endemic species; pollen limitation; reproductive success; reproductive system; secondary pollen presentation

## Introduction

The reproductive patterns are one of the key factors leading to the actual abundance, distribution and genetic diversity of organisms. In flowering plants a highly diverse array of floral traits and reproductive systems evolved varying from obligated cross-fertilization to obligated or promoted self-fertilization, with each strategy presenting selective vantages and disadvantages (reviewed by Takebayashi and 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. While selfing presents selective advantages, as reproductive assurance (during colonization processes, bottlenecks and scarce or unreliable pollinators) and twofold transmission of genes, crossing will increase genetic diversity reducing the risk of inbreeding depression. Therefore, it is important to search for distinct biological attributes of narrow endemic plants. For example, Murray *et al.* (2002) observed that, at a spatial scale, these species generally presented smaller flowering periods, and Lavergne *et al.* (2004) reported fewer and smaller flowers, less stigma-anther separation and lower pollen ovule 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 (*e.g.*, Gitzendanner and Soltis 2000; Hannan and Orick 2000; Cole 2003). This pattern does not seem to result from a predominant autogamic system within rare species, as it was independently observed in both self-compatible and self-incompatible species (Hamrick and Godt 1996), but it 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, which difficult the identification 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 endemisms and a significant representation in diversification centres as defined by the IUCN and WWF programs (Paiva 1998). Despite the conservation importance of this genus, few studies have been performed so far and the available information on its reproductive ecology is scarce. Several reproductive strategies have been described or assumed for *Polygala* species. Generally, individuals are described as entomophilous with specialized flower morphology (Faegri and van der Pijl 1979; Brantjes 1982), elaborated mechanisms of secondary pollen presentation (Ladd and Donaldson 1993; Yeo 1993; Paiva 1998) and flower ecology and pollination mechanisms parallel with those observed in Fabaceae (Brantjes

and van der Pijl 1980; Westerkamp 1999). Nevertheless, due to androecia and gynoecia morphology and disposition (see Figures in Paiva 1998), auto-pollination is assumed as the most common phenomenon (Venkatesh 1955, 1956; Dube 1962; Miller 1971), frequently occurring by delayed selfing (Lack and Kay 1987; Norderhaug 1995; Weekley and Brothers 2006). Additionally, several species produce chasmogamous and cleistogamous flowers, ensuring sexual reproduction and the possibility for crossing (James 1957; Ferrara and Quinn 1985, 1986; Weekley and Brothers 2006). These different reproductive strategies will result in different outputs that directly affect the population dynamics, with their understanding being of major importance to assess the status of rare species and develop management strategies (Schemske *et al.* 1994).

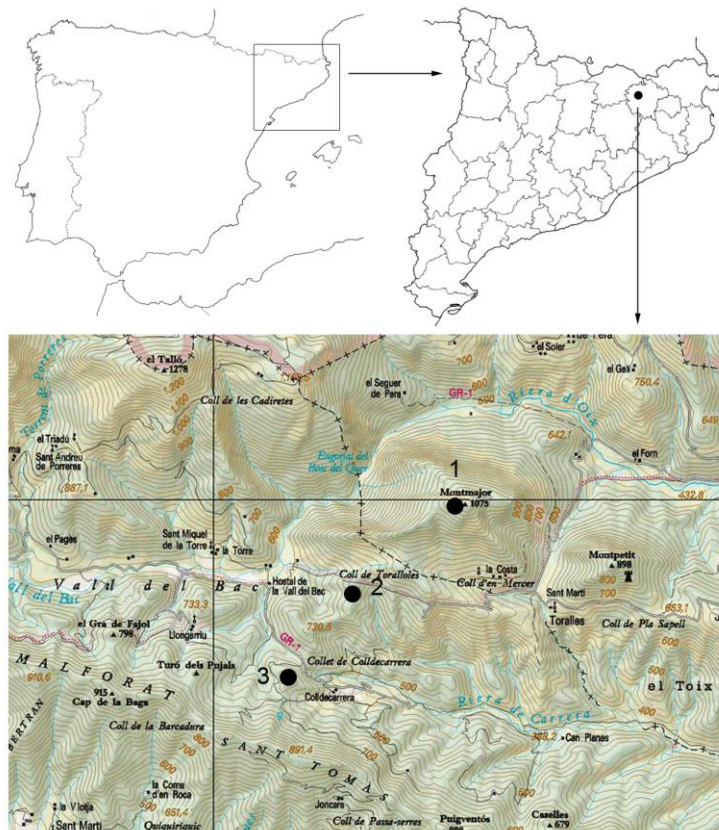
*Polygala vayredae* Costa (Polygalaceae) is a narrow endemic species from oriental pre-Pyrenees, present only in an area of approximately 12 km<sup>2</sup>. According with the IUCN categories, a preliminary endangered status has been given (vulnerable; VV. AA. 2000) and it is hypothesized that this species is a relict that survived through the Quaternary glaciations in restricted habitat pockets, where it currently occurs (Bolós 1946). 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 the present work we studied the flower biology and breeding system of *P. vayredae* to evaluate how they affect the reproductive outcome in natural populations. Three questions emerged: (1) how the flower functions as a sexual reproductive entity? (2) Does the plant depends on pollinators and/or presents an alternative mechanism for reproductive assurance? (3) What are the consequences of this on the reproductive success of natural populations? For this we studied flower morphology and floral rewards, both directly involved in plant-animal interactions, and examined the male and female functioning along flower lifespan 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 along two 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 information we intend to contribute to the knowledge of reproductive strategies in *Polygala* genus and provide relevant know-how that is essential for a correct management and conservation of *P. vayredae*.

## Materials and methods

### Plant and study area

*Polygala vayredae* is a small shrublet, annually sending up shoots from a rootstock forming 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. *P. vayredae* occurs in an area of approximately 12 km<sup>2</sup> in Alta Garrotxa, Girona (Catalunya, Spain, UTM DG57 and DG58; Fig. 2.1.1). The largest populations are observed in pine forests 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, 1,070 m, exposed rocky slopes; (2) Serrat dels Boixos, 750 m, open pine forest; and (3) Colldecarrera, 630 m, open pine forest (Figs. 2.1.1 and 1.1). Flower samples (preserved in ethanol 70%) and reproductive success data were collected in the three populations during spring of 2004 and 2005. The remaining experiments were performed in Colldecarrera population during spring of 2005.



**Fig. 2.1.1** Distribution of *Polygala vayredae* and location of the studied populations: 1 – Montmajor, 2 – Serrat dels Boixos and 3 – Colldecarrera (regional topographic map 1:50,000) in Alta Garrotxa, Catalunya, Spain (Iberian Peninsula).

### *Flower morphology*

Morphometric analyses were performed on 78 flowers from Coldecarrera population by collecting the following measurements: angle of flower presentation, and length of wing-sepals, corolla, corolla tube and keel appendage (Fig. 2.1.2B). Moreover, whenever nectar robbing occurred (*sensu* Inouye 1980), robbery signs and ovary damage were recorded for every flower. The presence of nectar guidelines was studied under ultra-violet light in flowers of recent herbarium vouchers (deposited at the Herbarium from University of Aveiro, AVE).

### *Flower lifespan*

Floral lifespan was studied by marking 23 flowers before opening. Flowers were then monitored daily, until senescence. Time of pollen release and relative position of style vs. anther were recorded to evaluate if delayed self-pollination occurred through style and stigma rotation.

### *Patterns of nectar production*

Daily nectar production was quantified in 37 flowers randomly selected along Coldecarrera population. The patterns of nectar production along flower lifespan were evaluated by quantifying the nectar in 12 flowers for each class of age (day -1, 0 or prior to anthesis, 1, 2, 3, 4, 5, 6, 7 and 8 days). In both treatments, the flowers were bagged 24 h before extracting the nectar. Nectar production was measured with a capillary micropipette and sugar concentration was determined (in w/w %) with a portable refractometer. The amount of sugar produced by each flower was calculated according with Prys-Jones and Corbet (1987).

### *Pollen and ovule production*

In order to preview the reproductive system of this species, the pollen ovule ratio (P/O) was determined (Cruden 1977). The number of pollen grains per flower was estimated in 10 anthers of bud flowers from distinct individuals of each population, according with 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 = 100x). The number of ovules was evaluated in the same flowers used for pollen counting. The number of aborted pollen grains was excluded from the P/O determination.

### *Stigma receptivity*

To determine stigma receptivity along 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 in 10 distinct individuals; the hand-pollinations were made in 10-12 flowers of each class of age (day 0 or prior to anthesis, 1, 2, 3, 4, 5, 6, 7 and 8 days); all the flowers were collected 24 h after pollination and harvested in ethanol 70% for subsequent examination of pollen tubes development. Pistils were cleared and softened with sodium hydroxide 8 N for 4 h, rinsed in distilled water and stained overnight with aniline blue 0.05% prepared in potassium phosphate 0.1 M (Dafni *et al.* 2005). Pistils were then placed on a microscope slide with a drop of glycerine 50% and squashed beneath a cover slip. Samples were observed through an epifluorescence microscope with a UV-2A filter cube (330-380 nm excitation). The number of pollen tubes growing throughout the stigmatic papillae and style was recorded. Percentages of receptive stigmas were calculated for each class of age.

### *Pollen germinability*

Pollen germinability along 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 class of age (as defined above); all the receptive flowers were collected 24 h after pollination and harvested in ethanol 70%. Pollen grains 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 fruit set, seed set, seed ovule ratio and seed weight was investigated. The following treatments were applied: (1) spontaneous autogamy, flowers were bagged with mosquito net to exclude insect interactions (n = 35 flowers); (2) obligated autogamy, flowers were bagged and pollinated with its 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 in 10 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). Every flower belonged to distinct plants. After 4-5 weeks, fruit and seed production were recorded and collected for seed weight measurement. Before weight measurement, seeds were dehydrated under natural conditions and maintained in a vacuum excicator with silica gel for 24 h.

Moreover, the self-incompatibility system was investigated in order to determine at witch level the inhibition of self-pollination occurs. Treatments 2, 3, 4 and 6 were repeated and 24 h after pollination, flowers were collected and harvested in ethanol 70% and pollen grains germination and pollen tubes development were examined under epifluorescence microscope (as described above).

#### *Indexes related with the breeding system*

With the results obtained in hand pollination experiments several indexes related with the breeding system were calculated. In order to discriminate the possible effect of dichogamy from genetic incompatibility, when necessary, self-pollination performance was determined using results of geitonogamous pollen transfer.

Selfing rate and inbreeding depression – The selfing rate ( $S$ ) was calculated according with Charlesworth and 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. Seed ovule ratio was chosen as indicator of reproductive performance.

Levels of inbreeding depression ( $\delta$ ) were determined by the relationship between the fruit set, seed ovule ratio and seed weight of geitonogamous-pollinated flowers ( $w_s$ ) and cross-pollinated flowers ( $w_x$ ) (Charlesworth and Charlesworth 1987):

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

Self-compatibility and self-fertilization indexes – The self-compatibility index (SCI) and the self-fertilization index (SFI) were calculated according with Lloyd and Schoen (1992): SCI is the proportion between the mean seed ovule ratio for manually self-pollinated flowers and the mean seed ovule ratio for cross-pollinated flowers; SFI is the proportion between the mean seed ovule ratio for spontaneous self-pollination transfer and the mean seed 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:

$$PPL = \frac{100 (PS - C)}{PS}$$

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

#### *Pollen limitation and fruit production*

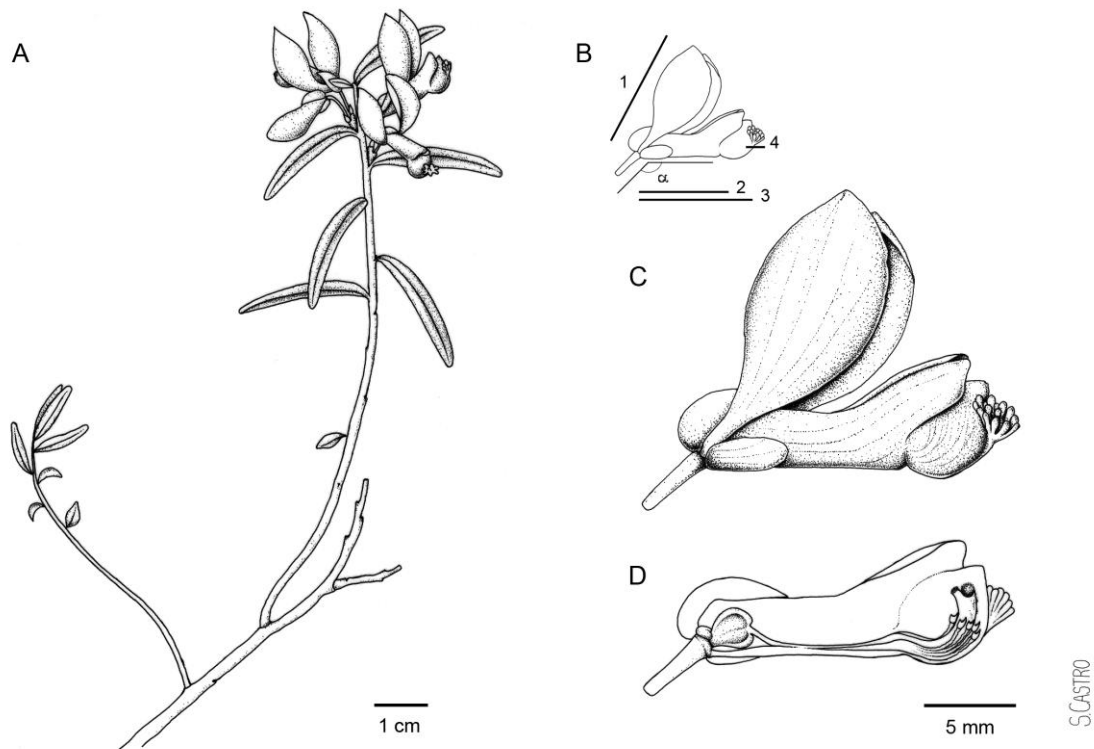
In order to evaluate if the reproductive outcomes are influenced by pollen limitation, along 2004 and 2005, pistils of 80 to 100 flowers from all populations were observed, and the presence/absence of pollen grains over the stigmas and pollen tube development were analysed (as described above).

To evaluate the constancy of the 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 were recorded and collected when mature.

#### *Statistical analysis*

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

The effect of the hand pollination treatments on the proportion of pollen tubes, fruit set and seed ovule ratio was analyzed with a  $\chi^2$  test for comparison of more than two proportions followed by a multiple comparison test for proportions (Zar 1984). Proportions of receptive stigmas along flower lifespan and proportions of pollen loads and pollen tubes, fruit set and seed ovule ratio among populations and years (categorical data approximated by a binomial distribution) were analysed with a logistic regression model with a link function (logit).



**Fig. 2.1.2** *Polygala vayredae*: (A) habit, (B) morphometric measures performed in the flowers [angle of flower presentation ( $\alpha$ ) and length of wing-sepals (1), corolla tube (2), corolla (3), and crest (4)], (C) complete flower and (D) open flower showing sexual organs disposition inside the corolla.

## Results

### Flower morphology

*Polygala vayredae* flowers appear in small axilar inflorescences of 1-3 flowers in a minute peduncle (Fig. 2.1.2A). The flowers are large ( $16.2 \pm 0.93$  mm long) and zygomorphic, with a remarkable superficial resemblance with Papilionaceae flowers (Fig. 2.1.2C). The calyx has five sepals, the basal two smaller, the upper one cup shaped, and the two laterals petaloid wings (wing-sepals) with  $15.9 \pm 0.99$  mm long, apparently involved in visitor advertisement. The corolla has three petals partially fused, the upper two almost completely fused forming a long corolla tube ( $14.0 \pm 0.72$  mm long) and the lower one forming a bowl or big cup (keel), extremely noticeable due to its sulphur yellow colour, connected with the corolla tube by a fold and functioning as a hinge; this lower petal has a fringed crest ( $2.4 \pm 0.46$  mm wide) that provides a good surface for insects foothold and is functionally analogous to the keel of Papilionaceae, enclosing and protecting the reproductive organs (Fig. 2.1.2D). 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 in 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. 2.1.2D) (Chapters 2.2 and 3.1). Flowers are exposed to potential visitors in an angle of  $143^{\circ} 47' \pm 10^{\circ} 60'$  with the petiole. No nectar guide lines were observed.

#### *Flower lifespan*

The flowers are opened for  $8 \pm 1.1$  days and during that time the style slightly curves 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. 2.1.2D). 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 of the movement of style during maturation, stigmatic papillae and anthers do not get in contact. Nevertheless, occasional selfing or self-interference could not be totally excluded as pollen is secondarily presented near the stigmatic papillae.

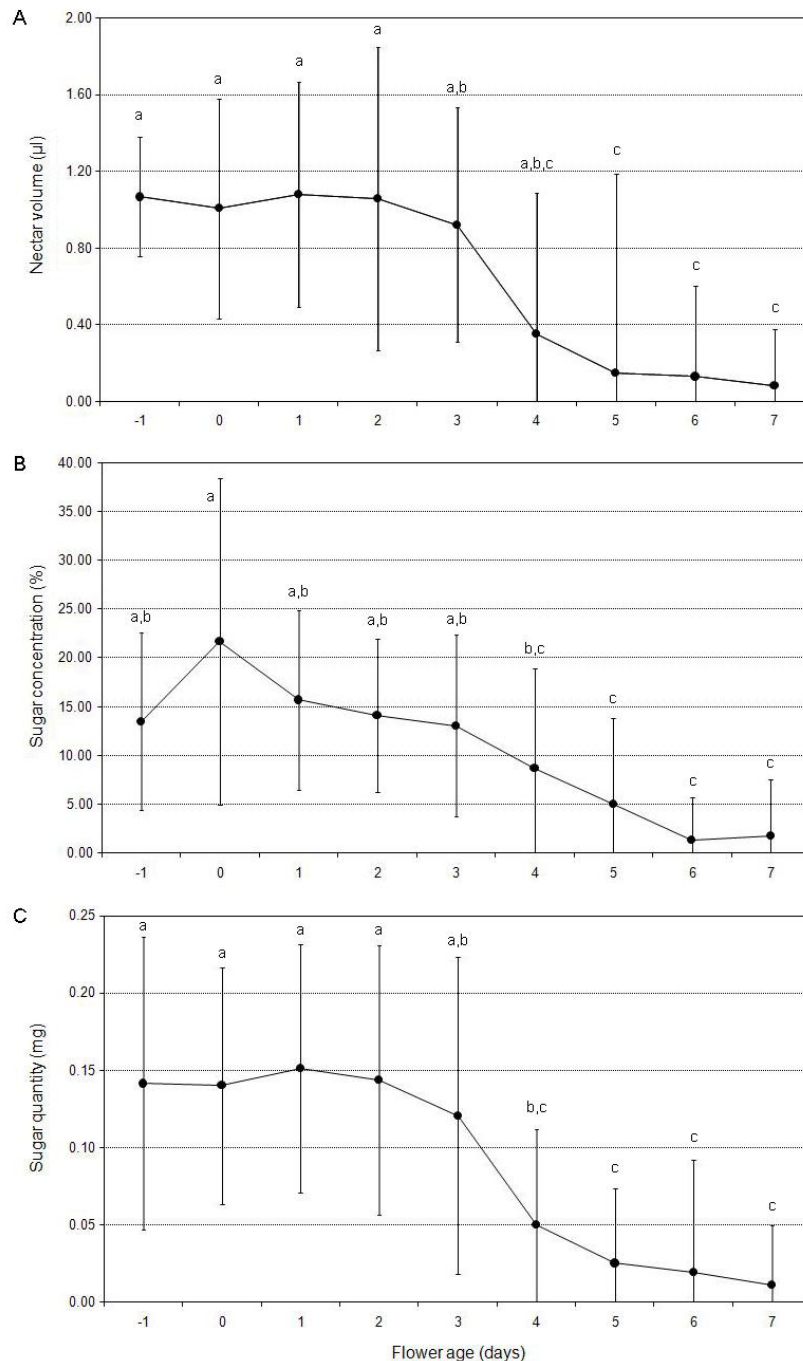
#### *Patterns of nectar production*

Nectar is produced at the base of the ovary in a conspicuous gland located between the two upper petals (Fig. 2.1.2D). The flowers produce  $1.9 \pm 0.86 \mu\text{l}$  of nectar with  $16.0 \pm 6.23\%$  of sugar, and are able to continue nectar production after removal of nectar.

In relation to flower age, nectar production starts before the anthesis and precedes pollen dehiscence. Although highly variable (Fig. 2.1.3), a clear pattern can be observed with a high nectar production in the first three days of the anthesis and an accentuated decreasing afterwards, in both nectar volume ( $H = 127.6$ ,  $P < 0.001$ ; Fig. 2.1.3A) and sugar amount ( $H = 161.3$ ,  $P < 0.001$ ; Fig. 2.1.3C). Regarding sugar concentration, the higher values were registered in the day of anthesis ( $H = 157.1$ ,  $P < 0.001$ ) coinciding with pollen dehiscence (Fig. 2.1.3B).

The nectar accumulates at the base of the corolla tube and in the upper sepal. Long-tongued bumblebees (*Bombus pascuorum* queens) were observed visiting *P. vayredae* flowers through a legitimate visit. Nectar and pollen robbery occurred and were clearly indicated by holes made by robbers in the upper sepal or corolla tube and keel,

respectively. The observation of flowers from morphometric analysis revealed the frequent occurrence of nectar robbing (70.5%), mainly by *Bombus terrestris* (Chapter 4.2), and in a lower extent of 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 the cases the ovary and nectar gland were damaged, respectively.



**Fig. 2.1.3** Patterns of nectar production along flower lifespan in *Polygala vayredae*. (A) Nectar volume (μl), (B) sugar concentration (%), and (C) sugar quantity (mg) according with flower age. Different letters reveal statistical differences at  $P < 0.05$ .

### *Pollen and ovule production*

*Polygala vayredae* flowers produce  $5428 \pm 1046.3$  pollen grains per flower, from which a variable percentage are aborted grains (0.8% - 56.7%). Each flower always produces two ovules. Excluding the aborted grains, flowers present a pollen ovule ratio (P/O) of  $2346 \pm 599.2$  (Table 2.1.1).

Differences in total pollen production per flower and P/O ratio were detected between populations ( $F = 3.53$ ,  $P = 0.043$ ;  $F = 8.44$ ,  $P < 0.001$ , respectively), decreasing from Montmajor to Colldecarrera and Serrat dels Boixos populations. No differences were found in the number of aborted pollen grains ( $F = 1.27$ ,  $P = 0.296$ ) (Table 2.1.1).

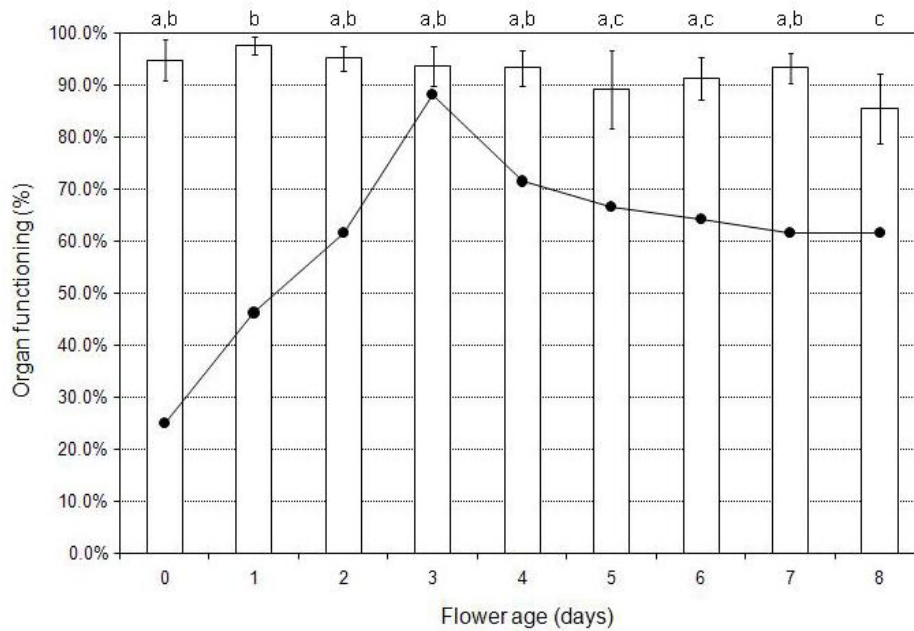
**Table 2.1.1** Total pollen, aborted pollen grains and pollen ovule ratio (P/O) produced per flower in three populations of *Polygala vayredae*.

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

Notes: The values are given as mean and standard deviation of the mean. Differences among populations were analyzed using a one-way ANOVA followed by a Tukey test. Different letters reveal statistical differences at  $P < 0.05$ . Pollen ovule ratio (P/O) was calculated excluding the aborted pollen grains.

### *Stigma receptivity and pollen germinability*

The observation of flower maturation seems to indicate a protandrous flower as the pollen is released in the style basket before anthesis. Nevertheless, the study of pollen viability revealed that pollen is able to germinate during all flower lifespan, only decreasing slightly in the last days of the flower lifespan ( $F = 5.76$ ,  $P < 0.001$ ; Fig. 2.1.4). On the other hand, the stigmatic 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 slightly decreases, although always maintaining a high proportion of receptive flowers until senescence ( $Wald \chi^2 = 3.5$ ,  $P = 0.627$ ; 61.5% of the flowers were receptive in the last days; Fig. 2.1.4).



**Fig. 2.1.4** Stigma receptivity (black dots), given as the proportion of flowers that enabled pollen tube growth along the style, and pollen germinability (open bars), given as the proportion of germinated pollen grains over stigmas, according with flower age in *Polygala vayredae*. Different letters reveal statistical differences at  $P < 0.05$  in pollen germinability.

#### Hand pollination experiments

Results of pollinator exclusion and hand pollination experiments are presented in Table 2.1.2. Pollen source affected significantly the fruit set and seed ovule ratio ( $\chi^2 = 110.6$  and  $137.6$ ,  $P < 0.001$ ). 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. Fruit set and seed ovule ratio in autogamous (14.3% and 7.1%, respectively) and geitonogamous pollinations (8.6% and 4.3%, respectively) were low. Additionally, the autogamous and geitonogamous treatments produced similar fruit set and seed ovule ratios indicating the lack of a dichogamous system that prevented mediated self-fertilization. This is in agreement with the results obtained for the male and female functioning (Fig. 2.1.4). Xenogamous and supplementary pollination treatments produced the higher fruit set (82.9% and 93.3%, respectively) and seed ovule ratio (51.4% and 66.7%, respectively). As a result, the estimated self-compatibility index ( $SCI = 0.084$ ) 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 ovule ratio ( $\delta = 0.916$ ) but relatively low for seed weight ( $\delta = 0.096$ ). Under natural conditions the fruit set (47.6%) and seed ovule ratio (36.9%) were significantly lower than

in the supplementary pollination treatment, indicating the occurrence of pollen limitation in Coldecarrera population during spring of 2005. Moreover, this is supported by a relatively high percentage of pollination limitation (PPL = 44.7%).

**Table 2.1.2** Number of pollen tubes, fruit and seed production and seed weight for *Polygala vayredae* flowers under different hand pollination treatments.

Hand pollination treatments	n	Number of pollen tubes†	Number of fruits (Fruit set)	Number of seeds (Seed ovule ratio)	Seed weight (mg) ‡
Spontaneous autogamy	35	0 <sup>a</sup> (0% <sup>a</sup> )	0 (0%) <sup>a</sup>	0 (0%) <sup>a</sup>	-
Obligatory autogamy	35	0.4 ± 0.82 <sup>a</sup> (17.1% <sup>b</sup> )	5 (14.3%) <sup>b</sup>	5 (7.1%) <sup>b</sup>	7.89 ± 0.942 <sup>a</sup>
Geitonogamy	35	0.5 ± 0.96 <sup>a</sup> (22.9% <sup>b</sup> )	3 (8.6%) <sup>b</sup>	3 (4.3%) <sup>b</sup>	
Xenogamy	35	5.0 ± 3.03 <sup>b</sup> (91.4% <sup>c</sup> )	29 (82.9%) <sup>c</sup>	36 (51.4%) <sup>c</sup>	8.73 ± 1.251 <sup>a,b</sup>
Supplementary pollination	30	10.4 ± 6.16 <sup>b</sup> (93.3% <sup>c</sup> )	28 (93.3%) <sup>d</sup>	40 (66.7%) <sup>d</sup>	9.43 ± 1.728 <sup>b</sup>
Control	42	8.9 ± 7.22 <sup>b</sup> (78.6% <sup>d</sup> )	20 (47.6%) <sup>e</sup>	31 (36.9%) <sup>e</sup>	8.17 ± 1.560 <sup>a</sup>
ANOVA		$H = 150.5^{***}$			$F = 6.19^{***}$
$\chi^2$ comparison for proportions		120.7 <sup>***</sup>	110.6 <sup>***</sup>	137.6 <sup>***</sup>	

Notes: Number of pollen tubes and seed weight (mg) are given as mean and standard deviation of the mean. The effect of pollination treatment in the proportion of flowers with pollen tubes, fruit set and seed ovule ratio was analyzed with a  $\chi^2$  test for comparison of more than two proportions followed by multiple comparison test (Zar, 1984). Differences in the number of pollen tubes per flower among treatments were analyzed with a Kruskal-Wallis one-way ANOVA followed by Dunn's method. Differences in seed weight among treatments were analyzed with a one-way ANOVA followed by a Tukey HSD test (GLM procedure). Different letters reveal significant differences at  $P < 0.01$ .

† The proportion of individuals with pollen tube development along the style is given in parenthesis.

‡ Due to the reduce number of seeds, mean seed weight of obligatory autogamy and geitonogamy was jointly assessed.

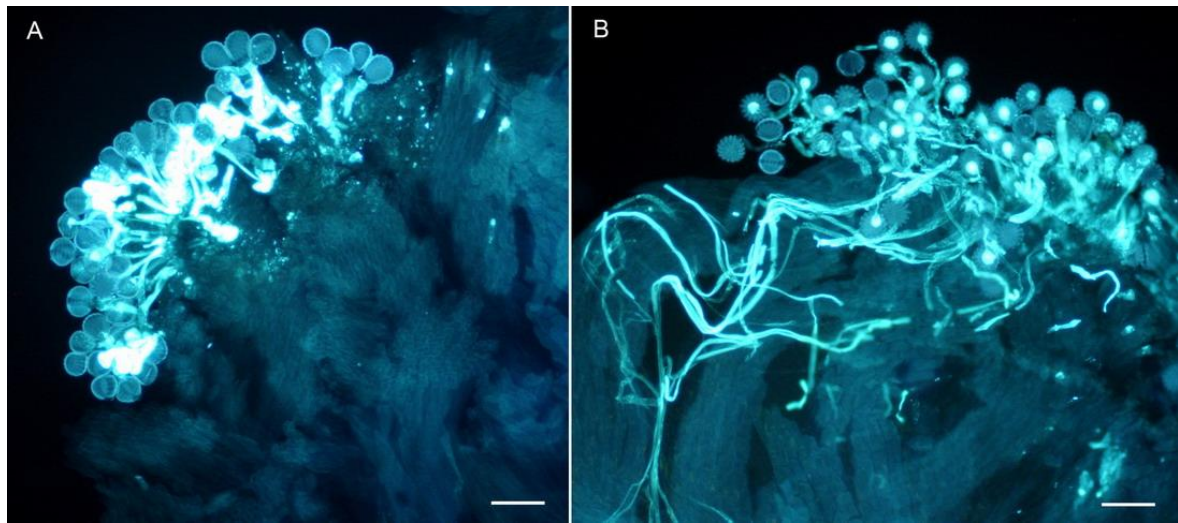
\*\*\*  $P < 0.001$

Regarding seed weights, differences were observed among pollination treatments ( $F = 6.19$ ,  $P < 0.001$ ). Supplementary pollination produced significantly heavier seeds than self-pollinations (autogamy and geitonogamy) and control ( $P < 0.01$ ). Autogamous and geitonogamous pollinations produced the lower seed weights (Table 2.1.2).

The observation of pollen tube development according with 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 2.1.2 and Fig. 2.1.5A). By other way, in xenogamous pollinations, pollen tube development along the style was observed in almost all the individuals (91.4%) and in higher number ( $5.0 \pm 3.03$  pollen tubes; Fig. 2.1.5B). The number of pollen tubes in supplementary pollinations and



control were slightly higher than in xenogamous pollinations, probably due to a higher quality of the pollen grains delivered by pollination vectors.



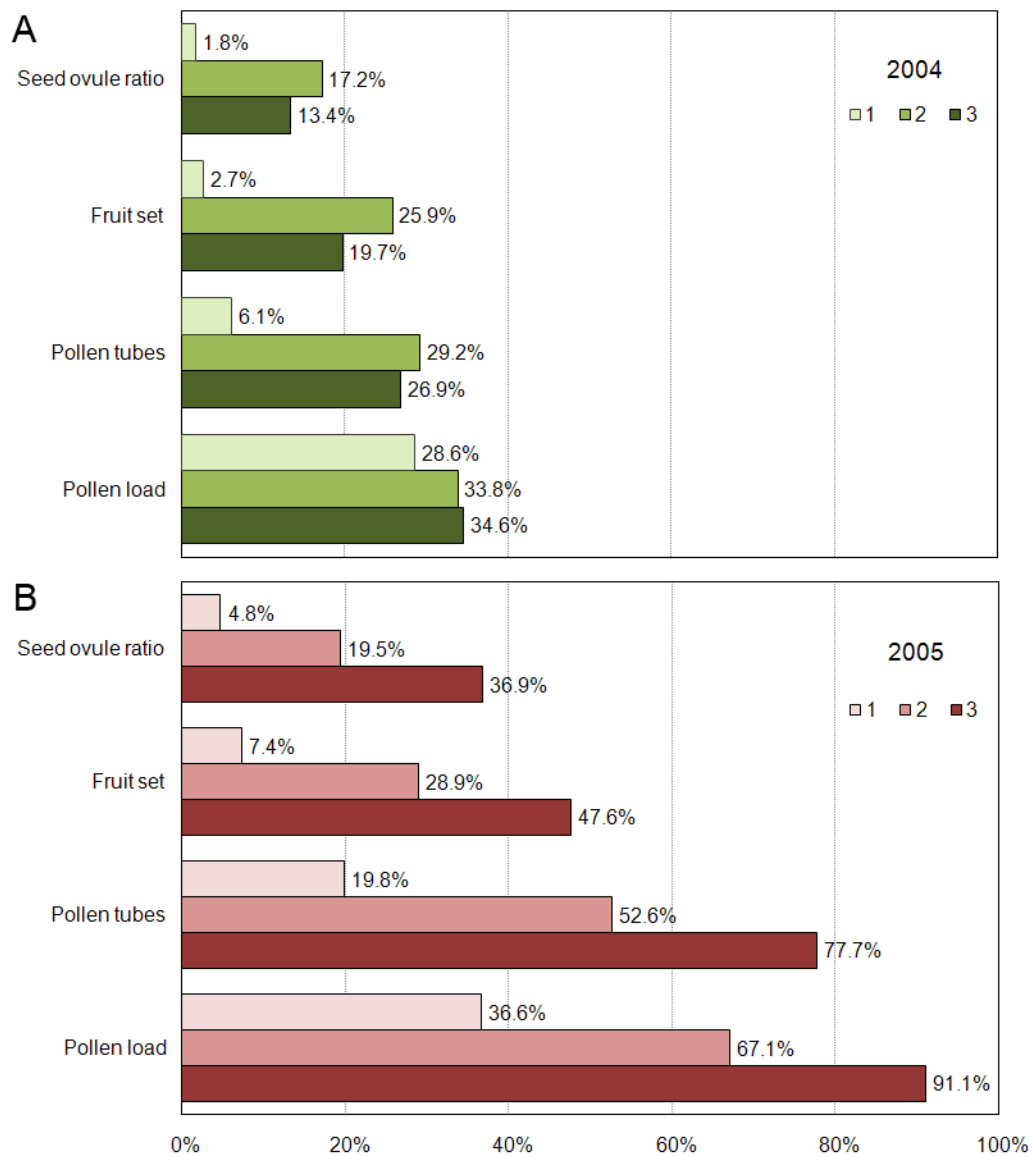
**Fig. 2.1.5** Pollen tube development at the stigmatic papillae and beginning of the style in *Polygala vayredae* after hand pollination experiments. (A) Geitonogamous pollination (B) Xenogamous pollination. Bars = 100  $\mu\text{m}$ .

#### *Pollen limitation and fruit production*

Results of pollen on stigma, pollen tube development and fruit and seed production are given in Figure 2.1.6. All parameters related to possible pollen limitation varied significantly among populations and years ( $\text{Wald } \chi^2 > 37.0$ ,  $P < 0.001$ ). Overall, values of any parameter were lower in all populations during 2004. Moreover, values for all analysed parameters decreased from Coldecarrera to Montmajor population during 2005.

Regarding the presence of pollen in the stigmas, the results showed that in general, the pollinator activity in this plant was low. This was evident during the year of 2004 in all populations (Fig. 2.1.6A) and during 2005 in Montmajor population, where only 28.6 to 36.6% of the flowers received visit(s) (Fig. 2.1.6B). Additionally, while during 2004 the estimated visitation rates were similar, during 2005 pollinator's activity varied significantly among populations (Fig. 2.1.6).

Secondarily, the quantity and quality of the delivered pollen also affected the reproductive outcome of *P. vayredae*. This was clearly evident in Montmajor population during 2004, where in flowers that received visit(s) pollen tube development along the style was only observed in 21.4% of the cases (*i.e.*, a decrease from 28.6% of visited flowers to only 6.1% where pollen tube development was observed, Fig. 2.1.6A), and in all populations during 2005 (Fig. 2.1.6B).



**Fig. 2.1.6** Pollen loads on stigma, pollen tube development, fruit set and seed ovule ratio of *Polygala vayredae* in three populations (1 - Montmajor, 2 - Serrat dels Boixos and 3 - Coldecarrera) along 2004 (A) and 2005 (B).

Finally, a significant reduction from the number of flowers presenting developed pollen tubes to flowers that set fruits was also observed, mainly during 2005 (e.g., in 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. 2.1.6B), although some differences were also recorded during 2004.

## Discussion

The flower is the structure directly involved with pollinator attraction and its morphological and functional traits affect the reproductive success of the plant. Thus, flower features may have great importance in narrow endemic species (Navarro and Guitián 2002). The floral morphology of *Polygala vayredae* is especially adapted for insect attraction. This species presents large zygomorphic flowers, with androecia and gynoecia concealed in the keel, advertisement wings and long corolla tubes, bright colours and a platform for visitor handle (Fig. 2.1.2). Furthermore, associated with this elaborated arrangement of floral pieces, several functional traits also reveal an adaptation for entomophily. Flowers have a large lifespan, which enhances the probability for receiving a visit (Ashman and Schoen 1996), produce abundant nectar like rewards for floral visitor and present a specialized mechanism of secondary pollen presentation (see Chapter 3.1).

Ashman and 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 along time (Ashman and Schoen 1994, 1996). According with this, long-lived flowers are selected when fitness accrual rates and floral maintenance costs are low, while 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 evolving differences in floral longevity. In a group of 11 species, Ashman and 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 constitutes important energetic costs (e.g., Southwick 1984; Pyke 1991), these results support the floral longevity model. *P. 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) seem to be the factors that explain the long lifespan of *P. vayredae* flowers (see Chapter 2.3).

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 not an exception. In *P. vayredae* flowers, while 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 the flower anthesis, when the higher nectar concentration is offered. Moreover, nectar composition also affects visitor behaviour and can generate different patterns of pollen exportation and reception (Thomson 1986; Harder and Thomson 1989). *P. vayredae* flowers produce abundant nectar with an unexpected low concentration of sugar for bumblebee pollinated flowers (e.g., Corbet 1978; Chalcoff *et al.* 2006; but see Percival 1965). Additionally, several authors demonstrated that flowers invest more in the exportation of the pollen through offering rewards, that in the pollen reception (Thomson 1986; Harder and Thomson 1989). Pleasants (1983) argues that variability on nectar production along flower lifespan is a way of offering different reward assemblages for flower visitors. Nectar production in *P. vayredae* flowers is higher during the male phase and decrease when female phase begins (see Fig. 2.1.4), in a strategy to improve male function through pollen exportation (Mitchell 1993). Nevertheless after the decrease in nectar production, as stigmas maintain a high level of receptivity and pollen grains sustain their germinability, cross-pollination may still occur with the other floral traits involved in pollinator's 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 by other ways are incapable to reach this reward (e.g., *Bombus terrestris* proboscides =  $5.8 \pm 0.34$  mm vs. *P. vayredae* corolla tube =  $14.5 \pm 0.78$  mm). This robbing attitude may not affect visitation by actual pollinators (*Bombus pascuorum* queens) as flowers are able to produce more nectar after removals, 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 in nectar production patterns, visitation rates, as well as the consequence of continuous resource removal in fruit production and seed weight (e.g., Chapter 4.3).

The efficiency of the insect visit can be improved by the mechanism of secondary pollen presentation (SPP). This mechanism evolved to spatially join the male and female functions, through pollen relocation in a specialized area of the gynoecia. It has been described as a strategy to improve accuracy in pollen removal and deposition, which will result in the enhancement of the male and female fitness (Howell *et al.* 1993; Yeo 1993; Ladd 1994). Different mechanisms may have evolved in parallel with SPP to reduce self-interference and self-fertilization, such as dichogamy and self-incompatibility systems (Lloyd and Webb 1986; Howell *et al.* 1993). Self-interference and self-fertilization have already been discussed by Brantjes (1982) in *P. monticola* H.B. & K. var. *brizoides* (St. Hil. & Moq.) Steyerm. and *P. vauthieri* Chodat. This author reinforced the importance of

the quality of the first visit in a precise pollen removal and deposition, such that when inefficiently done, auto-pollination (if a mechanism that prevents selfing is absent) or clogging of the stigmatic papillae with own pollen are prone to occur. On the other hand, parallel with SPP, mechanisms of delayed self-pollination could have evolved. Delayed selfing allows outcrossing when pollinators are present and provide reproductive assurance when pollinators are scarce or unpredictable (Seed *et al.* 2006). This mechanism seems to occur within Campanulaceae and Asteraceae by means of stigmatic lobes curvation (Faegri and van der Pijl 1979; Cerana 2004), and in several species of Polygalaceae by means of enclosed fertile and sterile stigmatic lobes (*e.g.*, *P. rugelii* Shuttlew. ex A.Gray, *P. nana* DC. and *P. setacea* Michx.; Weekley and Brothers 2006).

The results of the hand pollinations showed that *P. vayredae* requires pollinator visits in order to produce fruits, as no fruit was set after pollinator exclusion (Table 2.1.2). Despite of the occurrence of SPP near the receptive papillae and contrarily to what is observed in other species of the genus, *P. vayredae* does not possess a mechanism of self-fertilization nor delayed self-fertilization that provides reproductive assurance when visitors are scarce or inefficient. Additionally, 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 grains inhibition for self-pollinated flowers. The occurrence of an auto-incompatibility system will promote outcrossing and prevent inbreeding depression (Silva and Goring 2001). A self-incompatibility system was also described for *P. arillata* Buch.-Ham. ex D.Don, where a fruit set of 2.3% was obtained after autogamy (Devy and 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 and Kay 1987; Norderhaug 1995; Weekley and Brothers 2006), although most of those were only based in flower morphology, instead of considering the functional aspects. In *P. vayredae*, despite of the presence of an incompatibility system that prevents auto-pollination, the degree of self-interference due to male and female proximity is unknown (see Chapter 3.1). The reproductive system of *P. vayredae* is in accordance with the estimated P/O value that suggests a reproductive system between facultative and exclusive xenogamic (Cruden 1977).

Pollen availability and quality is considered one of the principal determinants of female reproductive success (Haig and Westoby 1988; Griffin and Barrett 2002) and pollen limitation has been shown to be widespread, especially in animal-pollinated species (Burd 1994; Larson and 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 Coldecarrera population during spring of 2005. Furthermore, the scarcity of pollen over the stigmas from three populations along two 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 seems to be the major factor affecting fruit production in Coldecarrera and Serrat dels Boixos populations, while in Montmajor, besides this factor the quantity and/or quality of the pollen also played an important role for the final reproductive outcome. This lower female fitness can result from the population size, as it was already shown that a small population size can reduce the number of compatible mates, especially in species with self-incompatibility barriers, and increase the relatedness among possible mates (Ågren 1996; Ward and 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 result in pollinator competition, increased heterospecific pollen delivery, and/or stigma clogging by heterospecific pollen (Campbell 1985; Gross 1996). Actually, in Montmajor population there are other species that begin to flower at the peak of *P. vayredae* flowering period, competing for the potential pollinators of this species, and diminishing the visitation rate of *P. vayredae* (S. Castro, unpublished data). In Serrat dels Boixos and Coldecarrera populations *P. vayredae* is the main species flowering. During 2005, pollinator activity was higher than in 2004 and significant differences were observed among populations. This resulted in a higher fruit and seed production in any population. Despite of this, several other features 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's behaviour, which can be influenced by plant density (e.g., Guitián *et al.* 1993; Grindeland *et al.* 2005). Coldecarrera and Serrat dels Boixos are large populations with dense clusters of *P. vayredae* and reduced seed dispersion (Chapter 5.1); 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.

The present study has shown that *P. vayredae* flower traits are actually indicatives of a xenogamic and enthomophylous flower, which are in accordance with the breeding

system of this species. The low fruit production in *P. vayredae* is largely due to scarce or unreliable pollinators and to pollen quality. Also, available resources could be a limiting factor that affects 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 dispersion and genetic structure are being developed to further understanding the biology of this species.

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## 2.2 Physical and nutritional constraints in pollen tube development of the endangered *Polygala vayredae* Costa (Polygalaceae)

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## **Abstract**

Low seed ovule ratios are observed in natural populations of *Polygala vayredae*, a narrow endemic species from oriental pre-Pyrenees. To understand potential limitations associated with pollination in this endemic species, pollen tube development along the pistil and the anatomy of the stigmatic region and style were investigated by fluorescent and light microscopy, respectively. Also, structural morphology of the stigmatic region was examined by scanning electron microscopy. The pollen grains that reached the stigmatic papillae entered in contact with a lipid-rich exudate and easily germinated. Despite of the large number of pollen grains that reached the stigmatic papillae, only a few pollen tubes were able to grow along the style towards the ovary. A hollow style was observed, with the stylar channel beginning few cells below the stigmatic papillae. At its beginning, the stylar channel was small in comparison with what was observed at the remaining levels of the style, and it was surrounded by highly metabolic active cells, rich in lipids. Furthermore, lipidic mucilage was detected inside the stylar channel. In subsequent style levels no major reserves were histochemically detected. The reduced intercellular spaces below the stigmatic papillae and the reduced area of the stylar channel at its beginning are suggested to physically constrain pollen tube development. In subsequent style levels the stylar channel can physically support a larger number of pollen tubes, but the lack of nutritional reserves cannot be disregarded. Finally, the number of pollen tubes entering the ovary was larger than the number of ovules, suggesting genetic interactions at this level that could play an important role in the final reproductive outcome of this species.

**Keywords** hollow style; pollen-pistil interaction; pollen tube development; stigmatic papillae; stigmatic exudate; stylar channel; style anatomy

## Introduction

After pollination and during the progamic phase, *i.e.*, the period of pollen tube growth through the pistil, intense and specific interactions occur between the male gametophyte and the pistil (Schrauwe and Linskens 1972; Herrero and Hormaza 1996). Pistils are especially suited to support pollen grain's germination and pollen tube growth: whereas, the stigmatic papillae supplies the environment for pollen germination (*e.g.*, Goldman *et al.* 1994; Edlund *et al.* 2004; and references therein) and the style provides the nutrition for pollen tube development (*e.g.*, Herrero and Dickinson 1979; Wang *et al.* 1993), the ovary seems especially involved with directional signalling for pollen tube growth (*e.g.*, Ray *et al.* 1997; Cheung and Wu 2001). Nevertheless, the pistil may also impose constraints to pollen tube development, either by self-incompatibility reactions or by a reduction in pistilar support (*e.g.*, Herrero 1992; Herrero and Hormaza 1996). Therefore, the knowledge of pollen tube development and pollen-pistil interactions is fundamental to perceive the reproductive outcome of plant species.

The anatomy of flowers from Polygalaceae family has already been extensively studied and used with taxonomic and phylogenetic purposes (Mukherjee 1961; Dube 1962; Milby 1976; Eriksen 1993; Prenner 2004). On the other hand, only a few studies have been developed on the pollination ecology (Brantjes and van der Pijl 1980; Norderhaug 1995; Weekley and Brothers 2006; Chapters 2.1, 3.1 and 4) and on the anatomical and morphological characteristics linked with implications on floral functioning and reproductive outcome (Venkatesh 1956; Westerkamp and Weber 1997; Chapters 2.1, 3.1, 4.1 and 4.3).

*Polygala vayredae* Costa (Polygalaceae) is an endangered narrow endemic species from oriental pre-Pyrenees, present in an area of approximately 12 km<sup>2</sup> only. It presents attractive flag blossoms (superficially similar to those of Fabaceae) specially arranged for insect pollination, but in natural conditions presents a low fruit set. An incompatibility system prevents self-fertilization after insect's visit, by blocking the pollen tube development of self-pollen at the stigmatic papillae (Chapter 2.1), and thus promoting outcross-pollinations. The pistil results from the fusion of two carpels and is constituted by a superior, bilocular ovary, with one pendulous ovule per locule, a hollow style, and a stigmatic area divided in two lobes with a specialized and diversified mechanism of secondary pollen presentation (for detailed illustrations see Chapter 3.1). During the study of the reproductive system of this species a reduced seed ovule ratio was observed, together with a reduced pollen tube development along the style when compared with the pollen load received in the stigma (Chapter 2.1). Furthermore, the style appears to suffer

a significant reduction in its diameter, from the top to the base (Castro 2005, personal observations), which can potentially impose limitations for pollen tube growth. Facing this observation, one may hypothesize that male gametophyte selection along the style occurs, either through a further recognition/rejection of pollen tubes or by physical and/or physiological constraints imposed by the style.

In the present study we investigated some of the possible causes involved in the selection of male gametophytes, namely, the physical and nutritional constraints of the female sporophyte. Primarily, pollen tube development along the style was studied in mature pollinated flowers to perceive the evolution of pollen tube growth along the style, and pollinations with different pollen loads were performed to assess if male gametophyte success was limited by the number of pollen grains that reached the stigmatic papillae. Then, pistil anatomy was investigated at the stigmatic papillae and at several levels of the style, in order to evaluate if its structure could limit pollen tube development by physical and/or nutritional constraints.

## **Materials and methods**

### *Plant material*

Flower samples of *P. vayredae* were collected in Colldecarrera population, La Garrotxa (Catalunya, Spain, UTM DG57), during the spring of 2005.

### *Pollen tube development along the style*

Open pollinated mature flowers from distinct individuals were randomly collected along the population and harvested in ethanol 70%. At the laboratory, pistils were softened with sodium hydroxide 8 N for 4 h, stained with aniline blue 0.05% overnight and mounted in a drop of glycerine 50% (Dafni *et al.* 2005). Samples were observed with an epifluorescence microscope equipped with a UV-2A filter cube (330-380 nm excitation), and the following parameters were assessed: total number of delivered pollen grains, number of germinated pollen grains, number of pollen tubes at the beginning, middle and end of the style, number of ovules with pollen tubes at the micropyle and diameter of pollen tubes. To confirm where pollen tube development occurs (in the style channel or in the transmitting tissue), manual sections of the style were obtained, stained with aniline blue and observed under an epifluorescence microscope. Pollen tube development along the style was analyzed with one-way ANOVA followed by a Tukey test for all pairwise multiple comparisons.

#### *A matter of pollen grains number?*

To understand if the number of pollen tubes growing along the style was dependent of the number of pollen grains that reached the stigmatic papillae, 87 flowers from distinct individuals were subjected by pollinators to different pollen loads (0-19, 20-39, 40-59, 60-79, 80-99, 100-199 and >200 pollen grains). Flowers were collected and harvested in ethanol 70%, 24-48 h after pollination. The development of pollen tubes in the style was evaluated following the procedure described above. The number of pollen tubes (at the beginning of the style) was represented as a function of the number of pollen grains over the stigma and compared with a one-way ANOVA followed by a Tukey test for all pairwise multiple comparisons. GLM procedures were used due to unbalanced data.

#### *Structure of the stigmatic region*

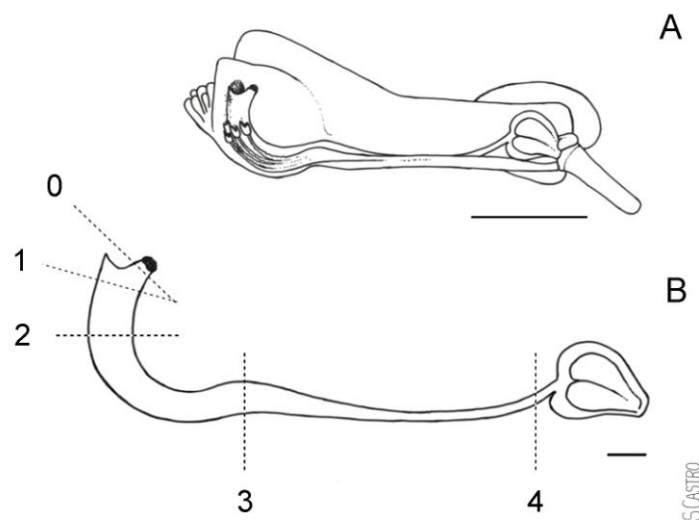
Mature flowers were collected and fixed in glutaraldehyde 2.5% [prepared in piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES) 1.25% buffer, pH 7.4]. The structure of the stigmatic region was studied through scanning electron microscopy (SEM). For this, samples were submitted to dehydration through successive aqueous ethanol solutions of increasing concentrations (50%-100%), and then ethanol was replaced through successive amiloacetate-ethanol solutions (1:3, 2:2, 3:1). Finally, samples were submitted to critical point, mounted on metallic stubs and coated with a gold/palladium film at high vacuum in a sputtering chamber. Samples were then observed with a scanning electron microscope, operating at 12 kV.

#### *Stigma and style anatomy*

Mature unpollinated flowers from five distinct individuals were collected and fixed in glutaraldehyde 2.5%. For light microscopy, samples were washed in PIPES buffer, immersed in osmium tetroxide 1.0% for 1 h and cleaned in PIPES buffer again. Then, samples were submitted to a dehydration process through an acetone series (50%-100%) and embedded in a graded low-viscosity epoxi resin (Embed-812). The following semi-thin sections (0.5-1.5  $\mu\text{m}$ ) were obtained using a glass knife: stigmatic papillae longitudinal and cross sections, sections below stigmatic papillae (0), sections below the stigmatic region (1), and sections at the beginning (2), middle (3) and base of the style (4) (Fig. 2.2.1). Sections were stained with periodic acid-Schiff (PAS) reaction for starch detection, with Sudan Black 0.3% (in ethanol 70%) for lipid detection, with blue of bromophenol 1% (in ethanol 95% saturated with  $\text{HgCl}_2$  10%) for protein detection and with toluidine blue 0.1% (in 1% of Borax 1% and Azur II 1%, 1:1) for general staining. Finally,



stained sections were observed under light microscopy and microphotographs were taken using a digital camera. Morphometric analyses were performed on photographs using ImageTool v. 3.0 for Windows. At the five levels of the style, the areas of the following structures were assessed: parenchyma tissue, vascular regions, style channel and intercellular spaces. The intercellular spaces were also evaluated just below stigmatic papillae. As at the level 0 the styler channel is not well limited yet, the area of the intercellular spaces in a 50  $\mu\text{m}$  radius was added to the channel area. Except for the proportion of intercellular spaces, differences among levels were evaluated with a Kruskal-Wallis one-way ANOVA on ranks, followed by a Tukey test for all pairwise multiple comparisons. The proportion of intercellular spaces was analysed using a one-way ANOVA.



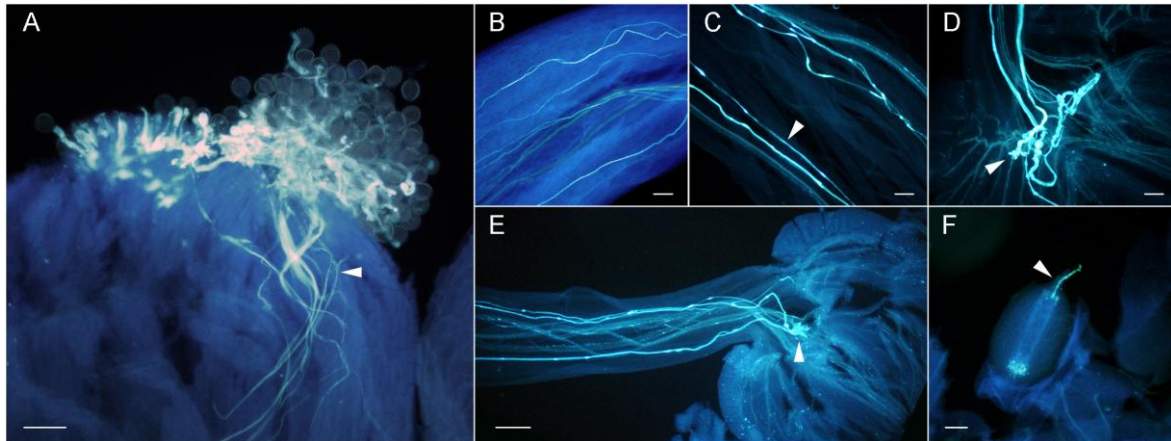
**Fig. 2.2.1** Schematic illustrations of the flower, androecia and gynoecia of *Polygala vayredae*. (A) Opened flower showing the stamens and the pistil disposition inside the corolla; (B) detailed illustration of pistil with studied sections highlighted. Studied sections: 0 - below stigmatic papillae, 1 - below the stigmatic region, 2 - beginning of the style, 3 - middle of the style, and 4 - base of the style. Bars: Fig. 2.2.1A = 5 mm; Fig. 2.2.1B = 1 mm.

## Results

### *Pollen tube development along the style*

Despite highly variable, the number of pollen grains deposited by pollinators on the stigma ( $67 \pm 79.0$  pollen grains) was usually larger than the required to fecundate the two ovules contained in the ovary. In general, all the pollen grains that reached the stigma germinated ( $98.4 \pm 2.87\%$  of the pollen grains) and the pollen tubes that were able to pass the stigmatic papillae grew throughout the style inside the styler channel. Pollen tubes presented a mean diameter of 11.3  $\mu\text{m}$  (mean area of 114.2  $\mu\text{m}^2$ ), but a high variability

was observed (standard deviation, SD = 4.32  $\mu$ m). They were observed growing highly directed throughout the style (Figs. 2.2.2A-C and E), but, once they entered the ovary, they frequently presented an erratic behaviour (Figs. 2.2.2D and E) with very few reaching the micropyle (Fig. 2.2.2F).

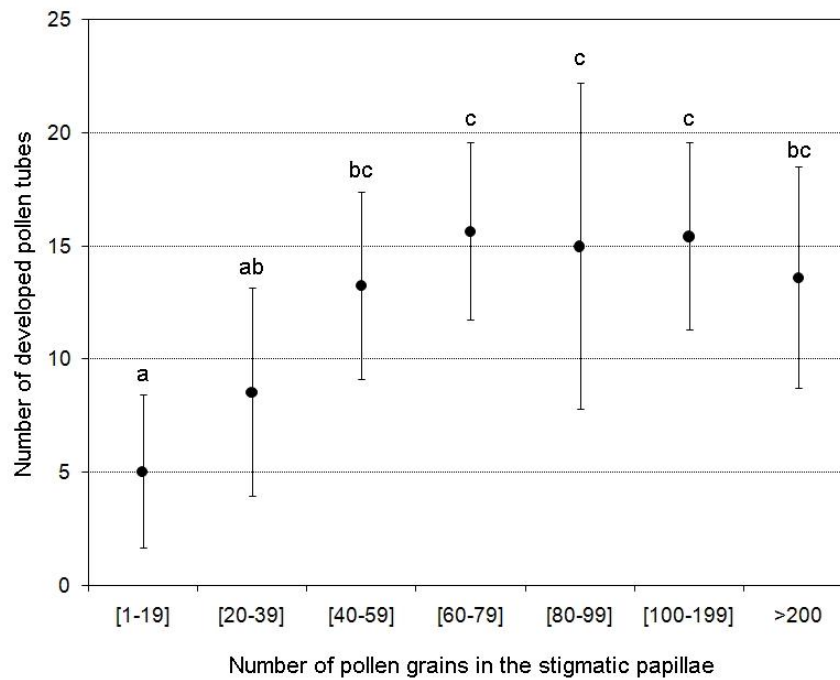


**Fig. 2.2.2** Pollen tube development along *Polygala vayredae* style after open pollination viewed under fluorescent microscopy. (A) Pollen grains over the stigmatic papillae and pollen tube growth below them (arrow); (B) pollen tube growth in the apical level of the style; (C) pollen tube growth in the middle level of the style (arrow - directed growing of the pollen tubes); (D) pollen tube behaviour at the entrance of the ovary (arrow - erratic behaviour of the pollen tubes); (E) pollen tube growth in the end of the style and entrance of the ovary (arrow - erratic behaviour of the pollen tubes); (F) pollen tube in the micropyle (arrow). Bars: Figs. 2.2.2A-D, F = 100  $\mu$ m; Fig. 2.2.2E = 200  $\mu$ m.

The number of pollen tubes growing along the style was low, from  $12 \pm 5.9$  at the beginning to  $9 \pm 5.1$  and  $8 \pm 5.0$  at the middle and base of the style, respectively. A significant decrease in the number of pollen tubes was observed from the beginning to the subsequent style levels ( $F = 8.25$ ,  $P < 0.001$ ). Despite the number of developed pollen tubes along the style was larger than the number of ovules (two ovules per flower), only 53.4% of the ovules presented a pollen tube at the entrance of the micropyle.

#### *A matter of pollen grains number?*

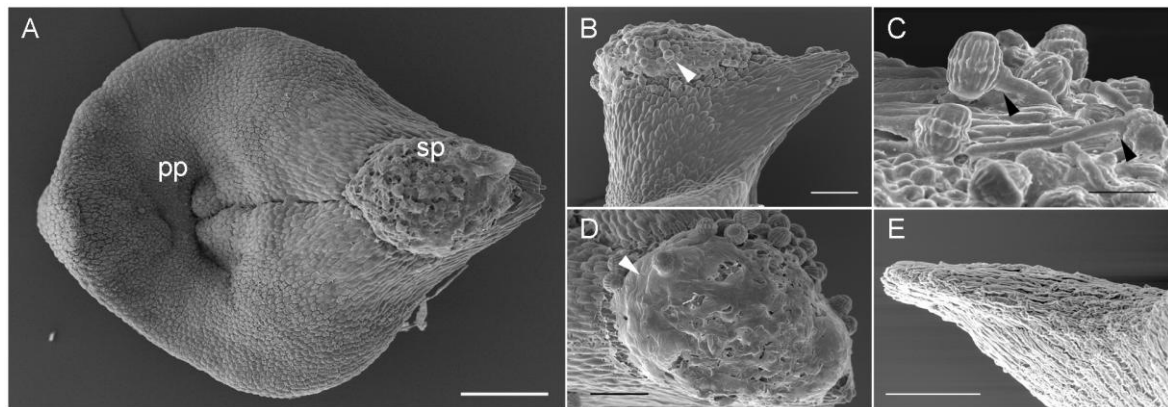
The number of developed pollen tubes as a function of the pollen load received on the stigma is represented in Figure 2.2.3. An increase in the number of pollen grains over the stigmatic papillae led to an increase in the number of developed pollen tubes, until a plateau was reached. At this level more than 60 pollen grains did not significantly increase the number of pollen tubes ( $F = 13.91$ ,  $P < 0.001$ ).



**Fig. 2.2.3** Number of developed pollen tubes in the style below the stigmatic papillae (mean and standard deviation of the mean) as a function of the number of pollen grains provided on the stigmatic papillae of *Polygala vayredae* flowers. Different letters reveal statistical significant differences at  $P < 0.05$ .

#### *Structure of the stigmatic region*

SEM allowed the observation of the structural morphology of *P. vayredae* stigmatic region (Fig. 2.2.4). In this species the stigmatic area is divided in two lobes: one anterior fertile, with wet stigmatic papillae, and another one posterior sterile, with a basket form (pollen presenter) specially adapted to pollen presentation (Fig. 2.2.4A). The stigmatic papillae are slightly elevated in respect to the sterile branch (Fig. 2.2.4B) and a conspicuous exudate production was detected (Fig. 2.2.4D). Also, pollen grains germination with pollen tube penetration in the stigmatic papillae was observed (Fig. 2.2.4C). The morphology of the epidermis was smooth in the branch where stigmatic papillae are elevated (Fig. 2.2.4B), and was highly ornamented in the pollen presenter, where pollen is released from the anthers to be presented to pollinators (Fig. 2.2.4E).



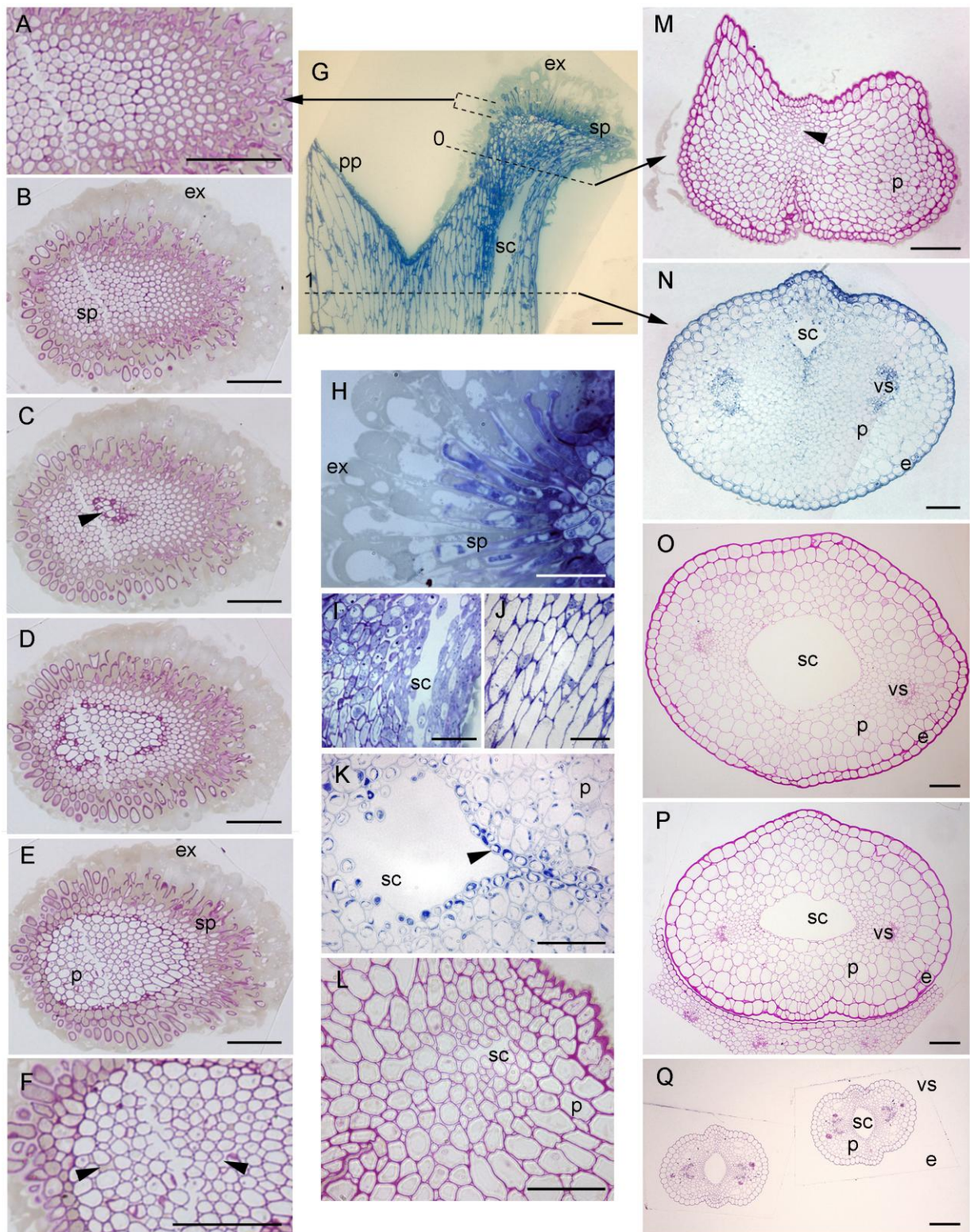
**Fig. 2.2.4** Structural morphology of *Polygala vayredae* stigmatic region under SEM. (A) stigmatic region, view from above; (B) stigmatic papillae (arrow), lateral view; (C) pollen grains germination (arrow); (D) stigmatic papillae with conspicuous exudates production (arrow), view from above; (E) extremity of pollen presenter. Legend: pp - pollen presenter, sp - stigmatic papillae. Bars: Figs. 2.2.4A-B = 200  $\mu$ m; Fig. 2.2.4C = 50  $\mu$ m; Figs. 2.2.4D-E = 100  $\mu$ m.

### Stigma and style anatomy

Histochemical and anatomical sections of the stigma and style are presented in Figures 2.2.5 and 2.2.6, and the main features are given in Table 2.2.1. In sequential cross sections of the stigmatic papillae (from the papillae, downwards; Figs. 2.2.5A-F) it was observed a tight disposition of the cells, with prominent exudate production (Figs. 2.2.5A-B). The receptive region of the stigma is formed by a unicellular layer of dense cells (Figs. 2.2.5G-H), which produced abundant mucilage mainly composed by lipids (Figs. 2.2.6A-B) where pollen grains stuck and germinated (Fig. 2.2.6A). The secretion and the papillae cell content were PAS negative, while the cell walls were PAS positive (*e.g.*, Figs. 2.2.5A-F). Also, no proteins were detected at this level.

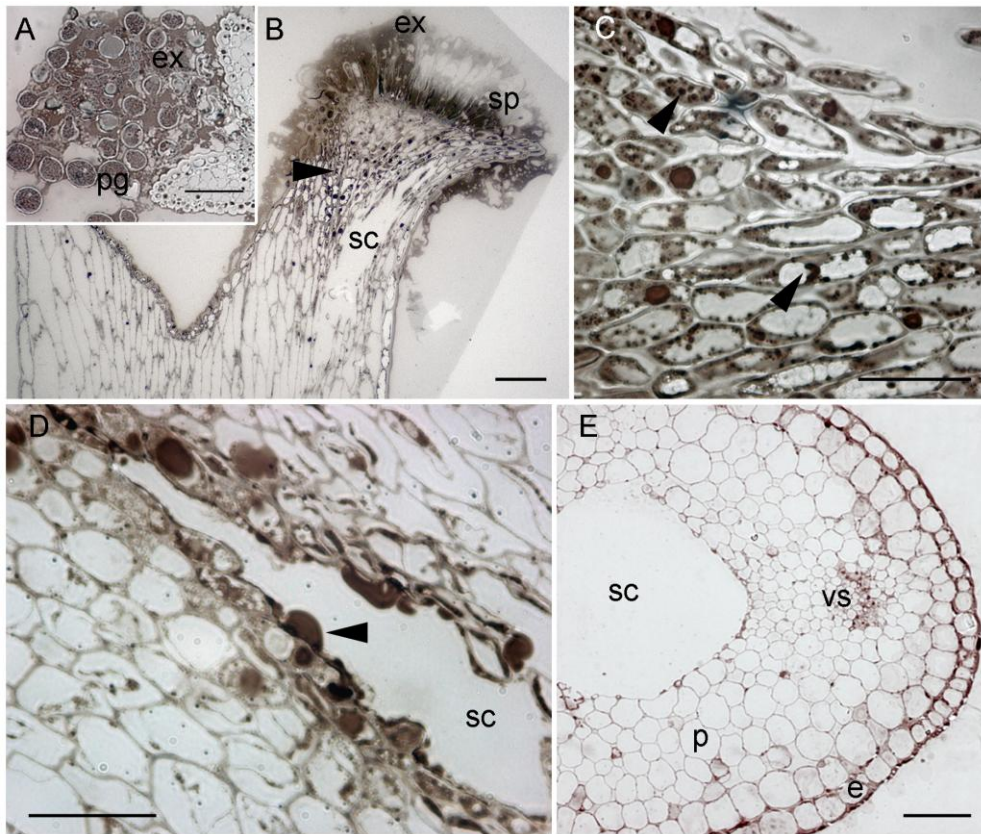
**Fig. 2.2.5** (right) Anatomical and histochemical characterization of the stigmatic region and style of *Polygala vayredae* stained with PAS (pink coloured) and toluidine blue (blue coloured). (A-E) Sequential cross sections of the stigmatic papillae, showing the stigmatic papillae, its base (arrow) and parenchyma cells below; (F) cross section just below stigmatic papillae, showing the intercellular spaces in the parenchyma (arrows); (G) longitudinal section of stigmatic region, showing the pollen presenter, the stigmatic papillae, the stylar channel and an active region below stigmatic papillae; (H) longitudinal section of the unicellular layer of stigmatic papillae; (I-J) cross section of the receptive branch in detail, just at the beginning of the stylar channel and 200  $\mu$ m below, respectively; (K) detail of the stylar channel below the stigmatic region, showing a cell layer of the epidermal cells at the abaxial side (arrow); (L) cross section at the beginning of the stylar channel, showing several spaces around (arrow); (M) cross section of receptive branch (level 0) at the beginning of the stylar channel (arrow); (N-Q) cross sections below stigmatic region and along the style (levels 1, 2, 3 and 4, respectively) represented at the same scale. Legend: e - epidermis, ex - exudate, p - parenchyma tissue, pp - pollen presenter, sc - stylar channel, sp - stigmatic papillae and vs - vascular strand. Bars: Figs. 2.2.5A-G, 2.2.5M-Q = 100  $\mu$ m; Figs. 2.2.5H-L = 50  $\mu$ m.





(Fig. 2.2.5)





**Fig. 2.2.6** Anatomical and histochemical characterization of the stigmatic region and style of *Polygala vayredae* stained with Sudan Black. (A) stigmatic exudate with adhered pollen grains, showing its lipid-rich composition; (B) longitudinal section of stigmatic region, showing the lipid-rich exudates over the stigmatic papillae and lipid accumulation in the parenchyma of the receptive branch (arrow); (C) detail of the receptive branch parenchyma, showing the lipid accumulation (arrows); (D) longitudinal section of the styler channel, showing lipid-rich secretions (arrow) and active cell layer of the epidermal type; (E) cross sections at the beginning of the style (level 2). Legend: e - epidermis, ex - exudate, p - parenchyma tissue, pg - pollen grains, sc - styler channel, sp - stigmatic papillae and vs - vascular strand. Bars: Figs. 2.2.6A-B, 2.2.6E = 100  $\mu\text{m}$ ; Figs. 2.2.6C-D = 50  $\mu\text{m}$ .

Once passed these papillae (Figs. 2.2.5C-F), a parenchyma tissue characterised by small intercellular spaces ( $26.2 \pm 31.82 \mu\text{m}^2$ ) is present (Fig. 2.2.5F). However, its total area of occupation ( $5.8 \pm 1.57\%$ ) was significantly higher than the one observed in the parenchyma tissue of adjacent levels (*i.e.*, levels 0 and 1;  $F = 142.4$ ,  $P < 0.001$ ; Table 2.2.1). Longitudinal sections of the stigmatic region revealed that the hollow pistil is a closed structure and that the styler channel starts in the direction of the stigmatic papillae ( $86.7 \pm 7.64 \mu\text{m}$  below) inside the receptive branch (Fig. 2.2.5G). Furthermore, in this area the parenchyma cells had dense cytoplasm (Figs. 2.2.5G and I) and accumulated lipidic reserves (strongly stained with Sudan Black; Figs. 2.2.6B and C), although no

carbohydrates or proteins were detected. Contrarily, the parenchyma cells at lower levels (levels 1-4) are larger, more vacuolated and depleted of reserves (Figs. 2.2.5J and 2.2.6E). Also, at the adaxial side of the beginning of the stylar channel, a cell layer of the epidermal type is observed (Fig. 2.2.5K) and, despite less conspicuous, a lipidic mucilage inside the channel was also detected at levels 0 and 1 (Fig. 2.2.6D). Throughout the following levels of the style (levels 2-4), the cells surrounding the wall of the channel did not reveal visible differences regarding cell contents when compared with the remaining cells of the parenchyma. Moreover, no carbohydrates (Figs. 2.2.5O-Q), proteins, lipids or mucilage production were detected at these levels (levels 2-4; Fig. 2.2.6E). Furthermore, the cells surrounding the wall of the channel appeared loosely arranged, mainly at the beginning (in the adaxial side; Fig. 2.2.5K) but also along the hollow style, only forming a defined epidermis at the end of the channel.

**Table 2.2.1** Description of the main anatomical features of *Polygala vayredae* style.

Level	Total area ( $\mu\text{m}^2$ )	Parenchyma ( $\mu\text{m}^2$ )	Stylar channel ( $\mu\text{m}^2$ )	Vascular boundaries ( $\mu\text{m}^2$ )	Intercellular spaces (%)
Below stigmatic papillae	-	-	-	-	$5.8 \pm 1.57^a$
Channel beginning (0)	$160,070.1 \pm 4,855.36^a$	$127,150.8 \pm 3,413.08^a$	$738.0 \pm 90.81^a$	-	$0.9 \pm 0.22^b$
Style level 1	$464,756.1 \pm 20,305.70^b$	$367,686.6 \pm 18,586.59^{bc}$	$7,778.7 \pm 378.06^b$	$25,990.5 \pm 1,411.76^a$	$1.1 \pm 1.32^b$
Style level 2	$675,080.4 \pm 94,844.87^c$	$490,165.7 \pm 59,566.57^c$	$68,479.2 \pm 21,915.09^c$	$13,395.6 \pm 2,320.75^b$	$4.7 \pm 0.68^a$
Style level 3	$603,711.4 \pm 129,073.02^c$	$432,102.3 \pm 88,470.71^c$	$53,624.5 \pm 21,914.19^c$	$14,841.3 \pm 7,395.56^b$	$4.9 \pm 0.60^a$
Style level 4	$88,423.9 \pm 18,254.87^a$	$53,577.7 \pm 10,592.27^a$	$7,684.9 \pm 1,915.49^b$	$5,342.3 \pm 709.34^c$	$15.4 \pm 0.36^c$
Comparison test	$H = 50.1^{***}$	$H = 63.9^{***}$	$H = 64.4^{***}$	$H = 44.2^{***}$	$F = 142.4^{***}$

*Notes:* Studied style levels: 0 - beginning of the stylar channel, 1 - below the stigmatic region, 2 - beginning of the style, 3 - middle of the style, and 4 - base of the style (for details see Figure 2.2.1). Values are given as mean and standard deviation of the mean. Differences among levels were evaluated with a Kruskal-Wallis one-way ANOVA on ranks, followed by a Tukey test for all pairwise multiple comparisons, except for the proportion of intercellular spaces where a one-way ANOVA was applied. Different letters indicate significant differences at  $P < 0.05$ ;  $***P < 0.001$ .

Morphometric analysis allowed a quantitative approach of the style anatomical sections (Table 2.2.1). Below the stigmatic papillae the parenchyma presents intercellular spaces that are irregularly distributed (Figs. 2.2.5D-F). From this point to level 0, the intercellular spaces get organized in the centre, where finally the stylar channel appears, and the

surrounding parenchyma gets highly compact (with few intercellular spaces; Table 2.2.1 and Figs. 2.2.5L-M). Afterwards, the intercellular spaces significantly increased along the style ( $F = 142.4$ ,  $P < 0.001$ ; Table 2.2.1). The stylar channel area was largely smaller at the receptive branch (level 0), increased until the middle of the style (up to level 2) and then reduced drastically to values similar to those of the initial style levels ( $H = 50.0$ ,  $P < 0.001$ ; Table 2.2.1; Figs. 2.2.5M and 2.2.5N-Q, with the latter represented at the same scale). Considering the area of the style channel below the stigmatic papillae (Table 2.2.1) and the mean area occupied by the pollen tubes ( $114.2 \pm 98.76 \mu\text{m}^2$ ), one can estimate that at this point (level 0) the stylar channel can physically support the simultaneous growth of only about six pollen tubes. In subsequent style levels (levels 1-4), from a physical point of view, the channel can potentially support a larger number of pollen tubes. Concerning the style area, it was smaller in the receptive branch (level 0), enlarged at level 1, 2 and 3, and finally it was significantly reduced from the middle (level 3) to the base of the style (level 4) ( $H = 50.1$ ,  $P < 0.001$ ; Table 2.2.1; Figs. 2.2.5M and 2.2.5N-Q, with the latter represented at the same scale). On the other hand, vascular boundaries were larger at the beginning of the style (level 0), after which they were significantly reduced until the base ( $H = 44.2$ ,  $P < 0.001$ ; Table 2.2.1).

## Discussion

In *P. vayredae* the pistil is sheltered inside the corolla and the stigmatic region gets exposed to the insect pollinators by the downward movement of the keel, which is mainly activated by the height of the insect (Chapter 3.1). The first half of the style is enclosed in the keel and is subjected to strong insect pressure during flower visit. This interaction may have driven selective forces towards a significantly thicker and robust structure, as the one observed in the present study. On the other hand, the second half (basal one) is protected in the corolla tube and is not submitted to any physical pressure, with the results evidencing a significantly thinner structure with more intercellular spaces.

The stigmatic region of *P. vayredae* is similar to those of other species of the genus, where a pollen presenter in form of a basket and a fertile branch with stigmatic papillae can be observed (e.g., *P. virgata* Thunb. var. *virgata*, Krüger and Pretorius 1997; *P. myrtifolia* L., Prenner 2004). This morphological adaptation is described as a way to accurately deliver and receive pollen, increasing the male and female fitness of the plant (Ladd 1994). Moreover, in the present work different epidermal ornamentations were observed in the stigmatic region of *P. vayredae*. These differences could be involved with self-pollen adhesion to the pollen presenter during secondary pollen relocation, and with



minimisation of self-pollen adhesion near the stigmatic papillae to avoid papillae clogging, as this species is self-incompatible (Chapters 2.1 and 3.1). Furthermore, an elevation of the stigmatic papillae in the fertile branch was also observed, with major implications in pollen receipt and avoidance of self-interference (Chapter 3.1; Ladd 1994).

When released from the anthers, most of the pollen grains are metabolically dormant and highly desiccated (ranging from 15 to 35% in water content; Heslop-Harrison 1979; Buitink *et al.* 2000). In species with wet stigmas, the exudate will influence pollen hydration, germination and pollen tube penetration through the stigmatic papillae (Goldman *et al.* 1994; Wolters-Arts *et al.* 1998). The stigmatic papillae of *P. vayredae* are active secreting cells that produce an exudate highly rich in lipidic substances. Lipids have been shown to play a key role in pollen-stigma interactions (e.g., Wolters-Arts *et al.* 1998; Edlund *et al.* 2004; and references therein). Through selection of several exudate compounds, Wolters-Arts *et al.* (1998) observed that lipids were the essential factor required for pollen tube penetration in the stigmatic papillae. These authors also proposed that the lipids present during pollen-stigma interactions were necessary to regulate the water uptake, which creates internal gradients involved in directional growth of the pollen tube; nevertheless, the mechanisms by which this happens remain unclear (for a review see Edlund *et al.* 2004). Similar lipid-rich exudates were described in *P. virgata* var. *virgata* (Krüger and Pretorius 1997), *Petunia* spp. (Wolters-Arts *et al.* 1998) and *Nicotiana sylvestris* Speq. (Kandasamy and Kristen 1987). Besides lipids, in *P. virgata* var. *virgata*, pectins and some proteins were further detected, while in *N. sylvestris*, proteins and carbohydrates have been also observed. Other components of the *P. vayredae* exudate may be present, but they could have been masked by the high abundance of lipids.

In *P. vayredae*, the stigmatic papillae appeared as the first barrier to pollen tube development due to a self-incompatibility system operating at this level (Chapter 2.1). After pollen tubes pass the stigmatic papillae, they penetrate in the stylar tissue. Anatomical data suggest that, just below the stigmatic papillae, pollen tubes would need to force throughout the parenchyma intercellular spaces and grow through the transmitting tissue until the stylar channel is reached. As in other species with hollow styles, pollen tube development along the style occurs inside the stylar channel (Leins and Erbar 2005; Reinhardt *et al.* 2007). In *P. vayredae* the number of pollen tubes that were able to pass the stigmatic papillae was low, dependent in some degree of a specific pollen load, but not limited by the pollen grains that reached the stigma afterwards.

Progressive reductions on the width of the transmitting tissue and in lipidic accumulations, from the stigmatic region to the base of the style, were observed in *P.*

*vayredae*. These features suggest changes in cell activity and in the reserves available through the stigmatic region and style. As pollen tube development along the pistil is heterotrophic (both in solid and hollow styles), changes in pistil structure and in the available reserves will play a crucial role in its development (Herrero and Hormaza 1996). In *P. vayredae*, the transmitting tissue immediately below the stigmatic papillae appears to physically constrict pollen tube development until the stylar channel is reached. At the top of the channel, pollen tube development could be further constricted, as the stylar channel and the surrounding intercellular spaces were very narrow and could only physically support the simultaneous growth of a reduced number of pollen tubes. In subsequent style levels (levels 1-4), the channel can potentially support a higher number of pollen tubes, at least from a physical point of view. However, from a nutritional standpoint, no major polysaccharide or lipidic reserves, or proteins accumulation were detected along the style. Thus, while in the receptive branch, around the beginning of the stylar channel, high cellular activity and reserves accumulation were observed, in the remaining levels, more vacuolated cells with less dense cytoplasm were present (a strong evidence for cells with low activities and depauperated reserves). Furthermore, at the beginning of the stylar channel, dense epidermal type cells showed strong staining for lipids and dense cytoplasm, suggesting high metabolic activity and production/release of lipid-rich mucilage to the interior of the channel. In hollow styles one or more layers of glandular cells lined along the stylar channel have been described. These secretory cells are responsible for the production of several components (Knox 1984; Cheung 1996) linked to pollen tube nutrition, guidance and adhesion (Sanders and Lord 1992; Cheung *et al.* 1995; Cheung 1996). Several experiments demonstrated the depletion of reserves within the transmitting tissue by the growing pollen tubes or the incorporation of components from the transmitting tissue or from the exudate into the pollen tubes (Labarca and Loewus 1973; Herrero and Dickinson 1979; González *et al.* 1996; de Graaf *et al.* 2003). Furthermore, the stylar secretion can also be involved with the assistance of pollen tube development along the channel, as proposed by Sanders and Lord (1989). Contrary to what is observed in other species, where the metabolism of polysaccharides is involved with pollen tube nutrition (Labarca and Loewus 1973; Herrero and Dickinson 1979), in *P. vayredae* the only histochemically detected reserves were of lipidic nature. Thus, despite some insights have been already given by the present work, further studies are essential to completely clarify how pollen tube nutrition occurs in this species.

The pistil appears to be designed to support/assist pollen tube development but, at the same time, it also encourages pollen-pistil interactions (Ray *et al.* 1997; Herrero 2000,

2001; Cheung and Wu 2001). In several studies a general reduction in the number of pollen tubes from the stigma to the ovary was observed (*i.e.*, pollen tube attrition) (Plitmann 1993; Erbar 2003), sometimes even after different pollination regimes (Hormaza and Herrero 1996; Smith-Huerta 1997; Ortega *et al.* 2002), indicating that genetic interactions play a major role in the control of pollen tube growth along the pistil. Furthermore, pollen tube attrition was observed to be significantly affected by the “quality” of the received pollen (*e.g.*, Cruzan 1989; Hormaza and Herrero 1996). In *P. vayredae*, around  $12 \pm 5.9$  pollen tubes were observed entering the style, and generally a larger number of pollen tubes ( $8 \pm 5.0$ ) than the number of ovules (two) reach the ovary. Nonetheless, usually only one of the ovules presented a pollen tube at the micropyle. Thus, besides the importance of the physical and physiologic constraints in pollen tube development, other factors as genetic interactions may be involved in pollen tube attrition along the pistil, ultimately determining the seed ovule ratio of this species. Indeed, in the present study, although the number of pollen tubes slightly diminished along the style, most of them were inhibited at the ovary. Pollen tubes were observed growing well directed throughout the style but, once at the ovary, their growth turned erratic, and frequently pollen tubes were not able to reach the ovules. The fairly large dimensions of the stylar channel suggest that there is no or reduced competition for space among pollen tubes and little contact between them and the transmitting tissue, possibly resulting in a reduction of incompatible reactions along the style. Nonetheless, as the path straightens to the ovary, interactions may be stronger, possibly leading to the observed erratic behaviour. Similar behaviours have been observed in several other species, with pollen tubes being arrested or suffering accelerations and decelerations in their growth at the entrance of the ovary (*e.g.*, *Prunus* spp., Herrero 2000; *Myrica rubra* (Lour.) Siebold & Zucc., Sogo and Tobe 2006). The available studies indicate that the ovary also appears to be another site where intraspecific and interspecific incompatibilities occurs (*e.g.*, Seavey and Bawa 1986; Williams *et al.* 1982) and that it may be even involved in compatible mating selection (Herrero and Hormaza 1996). Moreover, as pollen quality can also play an important role in male-female interactions and as, in the present work, pollinations were performed by insects with pollen being of unknown origin, further studies are needed to clearly understand the pollen tube dynamics in this species.

In the present study it was shown that several factors are involved in pollen tube development along the pistil of *P. vayredae*. First, strong interactions occur at the stigmatic papillae where the lipid-rich exudates trigger pollen grains germination, and a self-incompatibility system blocks self-pollen tubes to pass through the stigmatic papillae.

Second, physical constraints may be imposed to pollen tube development by the reduced intercellular spaces below the stigmatic papillae and by the reduced area of the stylar channel at its beginning. This physical constraint arise another topic, gametophyte competition, as the faster pollen tubes will be the ones reaching the stylar channel. Third, in subsequent style levels, despite the channel can physically support a larger number of pollen tubes, the lack of reserve accumulations could be one of the restraining factors involved in pollen tube development along the style. Finally, the number of pollen tubes entering the ovary was larger than the number of ovules, suggesting that genetic interactions play a detrimental role at this level. Additional studies involving sets of visited and unvisited flowers and controlled pollination experiments are needed to understand the dynamics of pollen tube development and reserve allocation along the style. Moreover, other factors may also be involved and despite new background information has been given on the present study, further investigations are still needed to clearly perceive the pollen tube pathway until fertilization is achieved.

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## 2.3 Effect of pollination on floral longevity variation and reproductive consequences in the long-lived *Polygala vayredae* Costa (Polygalaceae)

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## Abstract

The effect of pollination on flower lifespan have been widely studied, but so far poor attention has been given to the reproductive consequences of floral longevity. In the present study, the narrow endemic long-lived *Polygala vayredae* was used to answer the following questions: (1) how male and female success affects the floral longevity of individual flowers in *P. vayredae*? (2) How the delaying of the moment of pollination affects fruit and seed production as well as seed weight in this species? Floral longevity was studied after experimental pollinations involving male and/or female accomplishment, bagging and open pollination. The reproductive costs of floral longevity were evaluated after pollination of bagged flowers with 2 to 18 days through the fruit set, seed ovule ratio and seed weight. The senescence of the flowers of *P. vayredae* was activated by pollen grains reception on the stigma, but no effect of pollen removal from the pollen presenter was observed. Nonetheless, a minimum longevity period of eight days during which the flowers remained open was detected, even after pollination and pollen dissemination occurred. We suggest that this period can be involved in the enhancement of male accrual rates, as the female accomplishment is generally achieved after the first visit. The observed variation in floral lifespan may result from the frequent unpredictability of pollinators in this species. Also, it appears to bear the necessary plasticity to adapt to the abundance of pollinators. The maintenance of flower for longer periods as a consequence of delayed pollination had a major impact in the reproductive success of the plant, with fruit set, seed ovule ratio and seed weight being significantly diminished with the increase of flower longevity. The results of this study constitute the first description of variation in floral longevity within *Polygala* genus and highlight the strong relationship between pollination and floral longevity, as well as the reproductive costs associated with this trait.

**Keywords** endemic species; flower longevity; lifespan; pollination; secondary pollen presentation

## Introduction

Floral longevity plays an important role in the reproductive ecology of plants. The length of time a flower remains open and functional influences the total number of pollinator's visits and the size of floral displays, affecting the amount and quality of the pollen received and exported by the flower (Primack 1985; Ashman and Schoen 1996; Harder and Johnson 2005) and thus, determining the plant reproductive assurance and the overall fitness (Rathcke 2003). Flowers that present an extended longevity increase the opportunity for gene flow through both pollen and ovules, but also require a high maintenance cost to sustain its functioning and attractiveness to pollinators (Ashman and Schoen 1997). Factors, such as water balance (Nobel 1977), nectar production (Southwick 1984; Pyke 1991; Ashman and Schoen 1997), respiration rates (Bazzaz *et al.* 1979; Werk and Ehleringer 1983; Ashman and Schoen 1994) and/or loss of gamete viability with the increase of flower age (Smith-Huerta and Vasek 1984), have been described as the main costs involved in flower maintenance. Considering that the available resources are limited, a conflict between flower maintenance and other plant functions, such as flower construction and fruit and seed production, could arise. Thus, from an adaptive perspective, floral longevity should reflect a balance between the costs of flower production and maintenance, and its reproductive achievements (Ashman and Schoen 1994, 1996; Schoen and Ashman 1995).

Flowering plants exhibit a high diversity in the floral lifespan, suggesting that floral longevity could represent a character adapted to the surrounding ecological conditions, where abiotic (*e.g.*, temperature or water availability; Primack 1985; Yasaka *et al.* 1998) and biotic factors (*e.g.*, pollinator visitation rates; Ashman and Schoen 1994), as well as intrinsic features (*e.g.*, breeding system; Primack 1985; Sato 2002) could have an important effect in the strength of floral longevity. The effects of pollination on variations of floral longevity have been evaluated on several plant species and, overall, the results revealed a decrease in floral longevity with male and/or female accrual rates, *i.e.*, with the accomplishment of flower function (*e.g.*, Stead and Moore 1979; Ishii and Sakai 2000; Stpiczynska 2003; Abdala-Roberts *et al.* 2007). Nonetheless, so far only a few studies have experimentally evaluated the reproductive consequences of variations in flower maintenance (Holtsford 1985; Ashman and Schoen 1997; Abdala-Roberts *et al.* 2007).

*Polygala vayredae* Costa (Polygalaceae) is a narrow endemic species from the oriental pre-Pyrenees, with large, long-lived and papilionate flowers attractive to several floral visitors. Although the flowers are long-lived, different lifespans have been observed during flowering season. Like other endemic species (*e.g.*, Lavergne *et al.* 2005; Rymer *et al.*

2005), it is frequently subjected to pollinator unpredictability and pollen limitation (Chapters 2.1 and 4.2). The elaborated structure of the flower (closed corolla) requires the visit of specialized long-tongued and heavy bees which are able to move downwards the keel and efficiently pollinate the flower. Additionally, the flowers of *P. vayredae* receive the visit from several inefficient visitors, like nectar robbers (e.g., *B. terrestris*) and nectar thieves (e.g., *Macroglossum stellatarum*) (*sensu* Inouye 1980). Despite *Polygala* flowers are specially suited for animal pollination, the available information on its functioning is still scarce (but see Chapter 2.1 and references therein). The objective of the present work was to study the causes of variation in floral longevity and its reproductive consequences in *P. vayredae*. For this, the following questions were addressed: (1) how male and female success affects the floral longevity in *P. vayredae* flowers? (2) How the delaying of the moment of pollination affect fruit and seed production as well as seed weight in this species? Experiments involving different pollination treatments and xenogamous pollination of flowers with manipulated longevity were performed. By addressing these issues, this study constitutes the first report on floral longevity within *Polygala* genus and contributes with further information on reproductive consequences resulting from the costs of floral maintenance, a largely neglected topic in floral longevity literature.

## Materials and Methods

### *Plant and study area*

*Polygala vayredae* is a small shrub, with an early flowering period (April to May), distributed in a narrow area in Alta Garrotxa, Girona (Catalunya, Spain, UTM DG57 and DG58). Annually, new ramets are produced from a rootstock leading to the formation of dense carpets of this plant. Flowers are developed in small axilar inflorescences of 1-3 units and, under natural conditions, are open for  $8 \pm 1.1$  days (Chapter 2.1). Pollen dehiscence occurs prior to anthesis with most of the pollen grains being secondarily presented in a sterile branch of the stigma (named pollen presenter; Chapter 3.1). This species strictly relies on pollination vectors to set fruits, with the long-tongued bees *Bombus pascuorum* queens and *Anthophra* sp. being its main pollinators. Pollinator scarcity and unpredictability were observed during the period 2004-2007, frequently leading to pollen limitation (Chapters 2.1 and 4.2).

The study was performed during the springs of 2006 and 2007 in Coldecarrera population at the natural protected area of Alta Garrotxa (UTM DG57, for details see Chapter 2.1 and Fig. 1.1).

*Effect of pollination on floral longevity*

To determine the effect of pollination in floral longevity the following experiments were performed on randomly selected individual flowers during the spring of 2006: 1) flowers were bagged and all the pollen removed from the pollen presenter (after successive recharges until no pollen remained to be presented; see Chapter 3.1), *i.e.*, male accomplishment; 2) flowers were bagged and hand pollinated with xenogamous pollen, *i.e.*, female accomplishment; 3) bagged flowers with pollen removed and hand pollinated with xenogamous pollen, *i.e.*, both male and female accomplishment; 4) bagged flowers without any treatment; and 5) open pollinated flowers. In these experiments, the bagging process was conducted before anthesis to prevent natural pollination and the flowers were tagged with the day of flower opening. The pollen was removed immediately after anthesis and the pollinations were performed in the day three (when the peak of stigmatic receptivity occurs; Chapter 2.1). Flowers were monitored daily and floral longevity recorded.

*Costs of floral longevity on female fitness*

The costs of floral longevity, obtained after pollination at different times, on the fruit and seed production and seed weight were evaluated during spring of 2007. For that hand pollinations were performed in several individual flowers at different lifetimes, in order to obtain flowers pollinated from day 2 to day 18, the maximum longevity observed. The day 1 was excluded as stigmatic receptivity was found to be below 50% (Chapter 2.1). Eight clusters of 1 m<sup>2</sup>, with several reproductive ramets and bud flowers, were protected with a mosquito net to avoid natural pollination. Flowers were monitored daily and tagged with the day of anthesis until a set of flowers from 2 to 18 days of age was pollinated (2-4 flowers with the same age were tagged per cluster). Only ramets presenting one flower were selected. All flowers were hand-pollinated with a fresh pollen mixture collected in at least 10 distinct individuals. The mosquito net was maintained until flower senescence. Fruit and seed production were recorded when mature, and seeds were collected for determining their weight. At the laboratory, seeds were dehydrated under natural conditions, maintained in a vacuum excicator with silica gel for 24 h and weighed in an analytical balance (0.01 mg precision).

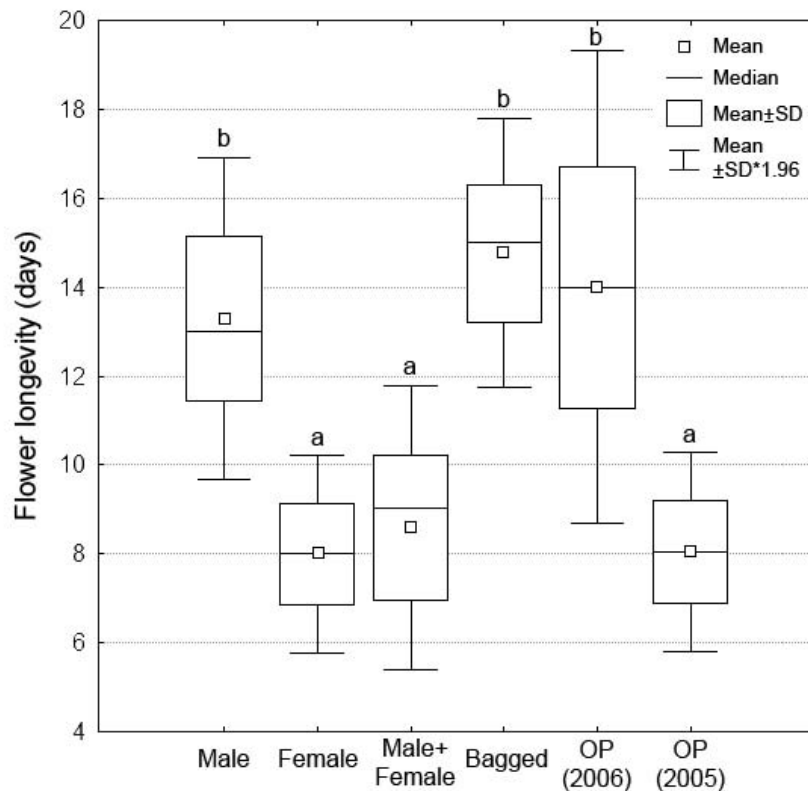
### *Statistical analysis*

Descriptive statistics were calculated for flower longevity and seed weight and are presented as mean and standard deviation of the mean. Differences in floral longevity among treatments and differences in fruit set, seed ovule ratio and seed weight according with flower age were assessed. The effect of the pollination treatment on flower longevity was evaluated with a Kruskal-Wallis one-way ANOVA on ranks using the Dunn's method for pairwise multiple comparison, also integrating the data on floral longevity obtained in the same population during the year of 2005 (Chapter 2.1). The effect of delayed pollination in the fruit set and seed ovule ratio (categorical data) was analyzed with a logistic regression model with a link function (logit) and approximated by a binomial distribution, while seed weight was analysed using a one-way ANOVA followed by a Tukey test (GLM procedure due to unbalanced data). The correlation between the moment of pollination and seed weight was investigated through the Pearson correlation coefficient.

## **Results**

### *Effect of pollination on floral longevity*

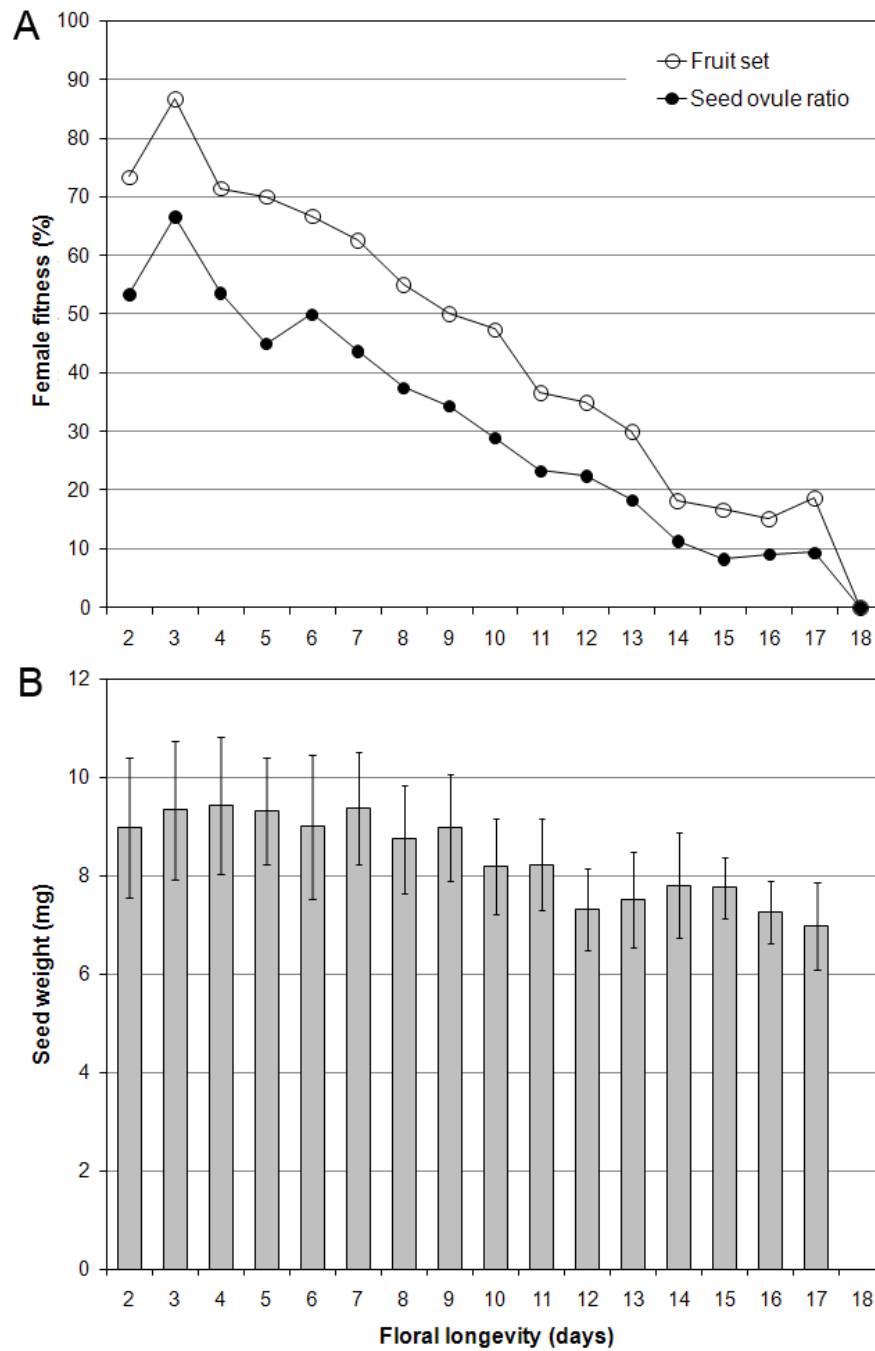
Pollination treatments significantly affected the longevity of the flowers of *P. vayredae* ( $H = 100.9$ ,  $P < 0.001$ ; Fig. 2.3.1). Nonetheless, this effect was not similar after male or female achievement: while floral longevity was significantly reduced when female function was accomplished (regardless of male function), it did not vary when male function was involved (bagged flowers and flowers with accomplished male function presented similar longevity; Fig. 2.3.1). Under natural condition, floral longevity varied significantly among years, with open pollinated flowers presenting longer lifespans during 2006 (despite variable, its floral longevity was similar to the one obtained in bagged flowers) than during 2005 (the floral longevity in this year was similar to when female function was accomplished). Also, despite the pollinations were performed at the day three of flower life (female fulfilment), a minimum of about eight days of floral longevity appears to occur regardless of male accomplishment.



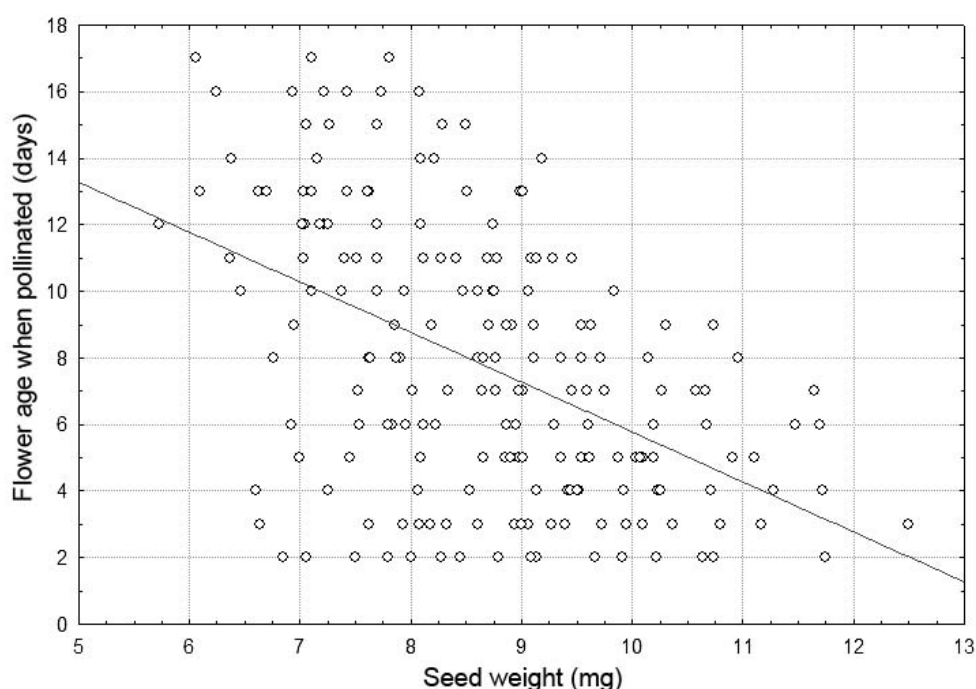
**Fig. 2.3.1** Longevity of *Polygala vayredae* flowers after different pollination treatments: Male - bagged flowers with all the available pollen removed; Female - bagged flowers hand pollinated with xenogamous pollen; Male + Female - bagged flowers with pollen removed and hand pollinated; Bagged - bagged flowers without any treatment; OP - open pollinated flowers from 2005 and 2006. Data from 2005 was obtained from Chapter 2.1.

#### Costs of floral longevity on female fitness

The fruit set, seed ovule ratio and seed weights obtained after pollination of flowers with different ages are presented in Figure 2.3.2. Fruit set and seed ovule ratio were higher when pollination was performed in the first days of flower lifespan and were significantly reduced with the increase of flower age ( $\chi^2 = 81.7$ ,  $P < 0.001$  and  $\chi^2 = 113.8$ ,  $P < 0.001$ , for fruit set and seed ovule ratio, respectively; Fig. 2.3.2A). Despite highly variable, a similar result was obtained with the seed weight ( $F = 4.90$ ,  $P < 0.001$ ; Fig. 2.3.2B). There was a significant and negative relationship between the age at which the flower is pollinated and the seed weight (correlation coefficient =  $-0.488$ ,  $P < 0.001$ ), *i.e.*, the increase of the flower age leads to a significant decrease in the seed weight (Fig. 2.3.3).



**Fig. 2.3.2** Costs of delayed pollination on female fitness: (A) fruit set and seed ovule ratio, and (B) seed weight obtained after pollination of *Polygala vayredae* flowers with different ages (2 to 18 days).



**Fig. 2.3.3** Scatterplot of the seed weight as a function of the age at which flowers of *Polygala vayredae* were pollinated (open dots) and fitted linear model (line).

## Discussion

Optimal floral longevity has been described as a trade-off between resource allocation to floral construction and floral maintenance (Ashman and Schoen 1994, 1996; Schoen and Ashman 1995). According with the model proposed by Ashman and Schoen (1994) and assuming a fixed resource availability, optimal floral longevities are 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 along time (reproductive success). As heritability is assumed to this floral trait, natural selection could play an important role in the optimization of floral longevity, with long-lived flowers being selected when fitness accrual rates and floral maintenance costs are low, and short-lived flowers selected when fitness accrual rates and floral maintenance costs are high. Thus, plants could adapt to different levels of pollinator activity through evolving differences in floral longevity (Ashman and Schoen 1994). In *P. vayredae*, the reliance on pollinators for seed production (Chapter 2.1) and the low visitation rates of efficient pollinators (Chapter 4.2) could be the most important factors explaining the long lifespan of its flowers. Furthermore, the floral duration appears to have some plasticity, as floral longevity varied significantly between years, with short longevities being observed during 2005 and long longevities during 2006. These results are in



accordance with the activity of effective pollinators and visitation rates reported for each year: the frequency of interactions between *P. vayredae* flowers and *B. pascuorum* queens, the main pollinator in Coldecarrera population, was clearly higher during 2005 (2.42, for 15 min following Herrera 1989) than during 2006 (0.09) (Chapter 4.2). Thus, it appears that flowers presented plasticity in floral longevity as a response to the abundance of pollinators, at least in some extent.

The observations above are also in accordance with the finding that floral longevity could respond to pollen dissemination and/or pollen receipt, revealing the necessary flexibility in this trait for optimizing the balance between reproductive output and resource efforts (Porat *et al.* 1994). In the present study it was shown a strong effect of pollen reception on the floral longevity in *P. vayredae*. Pollinated flowers, regardless of having their pollen removed, live for shorter periods than bagged flowers or flowers where only the pollen was removed. On the other hand, the fulfilment of the male function did not affect floral longevity. Similar effects of stigmatic pollen reception, regardless of pollen removal, on floral senescence were already observed in several other species (*e.g.*, Ishii and Sakai 2000; Luyt and Johnson 2001; Stpiczynska 2003). Experimental studies indicate that an increase of endogenous ethylene production is generally involved with corolla wilting and abscission (*e.g.*, Nichols *et al.* 1983), after a series of pollination-induced signals being generated within the floral tissues and transmitted through the style (*e.g.*, Stead and Moore 1979; Shibuya *et al.* 2000). Furthermore, the amount of pollen received also appears to play a major role as the amount of ethylene produced by the flower as been positively correlated with the amount of pollen received in the stigma in some species (*e.g.*, Hill *et al.* 1987; Stead 1992).

However, while the shortening of floral duration as a result of pollen reception is frequently observed (see references above), an effective shortening of floral longevity after pollen removal is rarer (but see Devlin and Stephenson 1984; Richardson and Stephenson 1989; Sargent and Roitberg 2000; Evanhoe and Galloway 2002). Thus, assuming that flowers react to pollen reception but not to pollen removal, and considering the advantages of plasticity in floral longevity (*i.e.*, reduction in maintenance costs, Harrison and Arditti 1976), Ishii and Sakai (2000) predicted that flowers will have a minimum longevity during which they do not abscise, even if most of their ovules are fertilised, so that male function can be enhanced. This was observed for *Erythronium japonicum* Decne., and similar patterns could also occur in other species which maintain their flowers for several days after being pollinated (Ishii and Sakai 2000, and references therein). In *P. vayredae* a minimum period of flower longevity also appears to be present

as flowers remained open for about eight days, despite female and male functions have been accomplished during the first days of the flower life. Because this plant secondarily presents its pollen near the stigmatic papillae, the first pollinator's visit is vital for successful pollination (female accomplishment) (Brantjes 1982; Chapter 3.1). On the other hand, as pollen can still be exported in subsequent visits, the minimum duration of floral longevity appears to be especially advantageous for a higher success of male fitness.

Despite the occurrence of a minimum longevity, the results above show that the reception of pollen accelerate floral senescence in *P. vayredae*. Furthermore, a trade-off between the moment of pollination (and thus, floral longevity) and reproductive output was observed. Fruit and seed production, as well as seed weight, decreased with the delaying of pollination, suggesting that the costs of flower maintenance reduced the reproductive outcome of *P. vayredae* plants. Similar experiments involving floral longevity manipulations were performed with *Calochortus leichtlinii* Hook. (Holtsford 1985), *Clarkia tembloriensis* Vasek (Ashman and Schoen 1997) and *Helleborus foetidus* L. flowers (L. Navarro, unpublished data), revealed comparable results. While in *C. tembloriensis* the maintenance of flowers for longer periods led to significant alterations in the size of subsequent flowers and of fruits (Ashman and Schoen 1997), in *C. leichtlinii* the maintenance of the first flower for longer periods led to a significant decrease of the seed production of the second flower (Holtsford 1985). In *H. foetidus*, longer floral lifespans resulted in both fewer and lighter seeds (L. Navarro, unpublished data). Also, in a different approach, a trade-off between floral longevity and flower construction-maintenance was observed in both *Cohniella ascendens* (Lindl.) Christenson and *Petrocoptis viscosa* Rothm., with the reduction of the number of flowers in the inflorescence leading to longer floral lifespans of the remaining flowers (Navarro 1996; Abdala-Roberts *et al.* 2007, respectively). Furthermore, Guitián and Navarro (1996) observed an increment in the seed production of the remaining flowers of the inflorescences after the elimination of the central flower on *Petrocoptis grandiflora* Rothm., revealing that the available resources can be re-distributed within the integrated physiological units (IPU's, *sensu* Watson and Casper 1984). Despite poor attention has been given to the reproductive costs and consequences of floral longevity, the few available studies, together with the present results, reveal the need to evaluate both gains and expenses of floral longevity at the level of IPU's structure in future studies of plant reproductive biology.

The present study constitutes the first report on floral longevity variation within the *Polygala* genus, revealing the role of stigmatic pollen reception on floral lifespan, as well

as the ability to extend or reduce floral longevity, within some limits, in response to the abundance of efficient pollinators (*i.e.*, reproductive fulfilment rates). Furthermore, the maintenance of the flowers for longer periods before pollination negatively affected the fruit and seed production as well as the seed weight, highlighting the reproductive costs of floral maintenance in offspring production.

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## Chapter 3

### Secondary pollen presentation

#### 3.1 How does secondary pollen presentation affect the fitness of *Polygala vayredae* (Polygalaceae)?

Chapter section submitted as an original article to SCI journal:

Castro S, Silveira P, Navarro L. 2007. How does secondary pollen presentation affect the fitness of *Polygala vayredae* (Polygalaceae)? *American Journal of Botany* (submitted).





## Abstract

Secondary pollen presentation (SPP) is the relocation and presentation of the pollen in floral structures other than the anthers, often found in close proximity to the stigma, and has been hypothesized as being an accurate mechanism of pollen transfer, although no experimental studies have been carried out to date. We examined the functioning of the pollen presenter (PP) and its efficiency in pollen dispersal in addition to female fitness and the degree of interference created by the self-pollen in *Polygala vayredae*, an insect-pollinated species with SPP. Micro-herkogamy, a mechanism used to reduce self-interference, was also evaluated. High pollen losses were observed during the secondary relocation of the pollen in the PP (49% of pre-collection pollen loss). The PP allowed the pollen to be exported and subsequent pollen losses were similar to those in species with primary pollen presentation. Despite the presence of a self-incompatibility system, the number of developed pollen tubes as well as fruit and seed production were significantly affected by the self-pollen interference created at the stigmatic papillae level. Micro-herkogamy, while reduced, correlated positively with female fitness. The SPP mechanism may in fact be an accurate system for pollen transport but it also has its costs. The effects of the close proximity of pollen presentation and reception sites on plant fitness and the occurrence of herkogamy were investigated experimentally for the first time in a species with SPP.

**Keywords** female fitness; herkogamy; pollen dispersal; pollen presenter; pollen relocation; Polygalaceae; self-incompatibility; self-interference

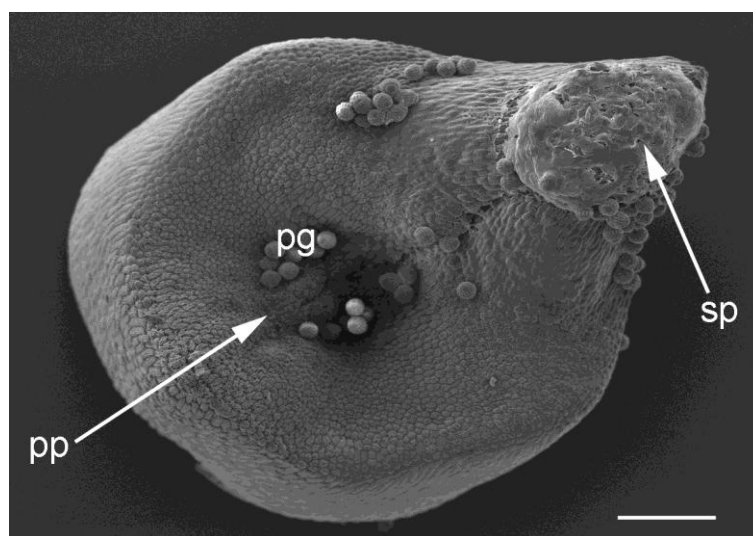
## Introduction

In several plant species pollen is presented in floral structures other than the anthers, either by simple deposition or by means of special expulsion mechanisms involving the pollen coming into contact with other floral parts. This floral mechanism is known as secondary pollen presentation (hereinafter called SPP; Faegri and van der Pijl 1979; Yeo 1993) and is the result of functional and/or adaptive features of the flower which enhance the placement of pollen onto the pollination vector (Inouye *et al.* 1994; Ladd 1994). The mechanisms through which pollen is secondarily presented are highly variable, with pollen being presented onto the style (Nyman 1993), in specialized regions of the style or stigma (Vaughton and Ramsey 1991; Westerkamp and Weber 1997) or even over the stigmatic area (Nilsson *et al.* 1990; Imbert and Richards 1993), usually before flower opening. Pollen is then exposed in the pollen presenter (hereinafter called PP) during floral development or the mechanism is triggered by the pollination vectors (*e.g.*, Brantjes 1982, 1983; Nyman 1993; Smith and Gross 2002).

Secondary pollen presentation has traditionally been described as a mechanism that enhances the efficiency and accuracy of pollen exportation and/or pollen reception, thus increasing the male and/or female fitness of the plant (Carolin 1960; Lloyd and Yates 1982; Ladd 1994). Nevertheless, very few studies have experimentally evaluated the effects of this mechanism on plant fitness (*e.g.*, Lloyd and Yates 1982; Imbert and Richards 1993; Nyman 1993). Moreover, it has also been suggested that a clash of interests may exist, as the close proximity of pollen-receiving and pollen-donating surfaces could result in self-interference, *i.e.*, a conflict between male and female functions (Webb and Lloyd 1986; Ladd 1994; Barrett 2002), with subsequent detrimental effects on plant fitness (*e.g.*, Cesaro *et al.* 2004; Kawagoe and Suzuki 2005; Waites and Ågren 2006). Several functional or adaptive floral traits, such as self-incompatibility, dichogamy or herkogamy, have evolved to avoid or minimize the effects of self-interference and thus improve outcrossing rates (Lloyd and Webb 1986; Webb and Lloyd 1986). Nonetheless, to date, self-interference has only been studied in species with primary pollen presentation and all the assumptions made regarding SPP are based mainly on morphological descriptions.

The species belonging to *Polygala* L. (the most representative genus of Polygalaceae comprising around 725 species; Paiva 1998) have been described as presenting a SPP mechanism in which pollen is released before anthesis in a PP located on a sterile branch of the stigma (*e.g.*, Ladd and Donaldson 1993; Westerkamp and Weber 1997; Fig. 3.1.1). However, there are some species that still present the ancestral gynoeceia with two carpels

and where this mechanism is absent (*P. persicariaefolia* DC.; Venkatesh 1956); in others the sterile stigma is reduced in size or has even disappeared (African *Polygala* sp.; Ladd and Donaldson 1993), and yet others where the PP has reappeared (*P. vauthieri* Chodat; Ladd 1994). Since the ancestral condition in Polygalaceae would seem to be the occurrence of two carpels, the PP most likely originated from the specialization and sterilization of one of the ancestral stigmas (Ladd 1994). The stigmatic region is highly diversified with structures having the shape of a basket, spoon, brush or hair crown, and in several species of this family, the PP is slightly displaced in relation to the stigma (for illustrations see Paiva 1998; Chapter 2.2). It has been proposed that species with SPP use a micro-herkogamy mechanism as a way to minimize the effects of self-interference due to the proximity of the self-pollen and the stigma (Ladd 1994). However, this theory has never been objectively investigated. Moreover, despite the fact that no studies targeting SPP in *Polygala* have been performed so far, the efficiency of pollen exportation/receipt by this mechanism has been questioned by Brantjes (1982) and Ladd and Donaldson (1993), since stigma clogging and/or self-pollination would appear to be difficult to avoid.



**Fig. 3.1.1** Morphology of the stigma of *Polygala vayredae* showing the pollen presenter (pp), the stigmatic papillae (sp) and several pollen grains (pg) (observed through scanning electron microscopy). Bar = 200  $\mu$ m.

Therefore the endemic species *Polygala vayredae* Costa (Polygalaceae) was selected to evaluate experimentally how the SPP mechanism affects plant fitness. Studies were conducted to determine the following: (1) PP functioning and the intrinsic pollen losses of the mechanism; (2) the efficiency of the PP in pollen dispersal; and (3) female fitness and the degree of self-interference. Furthermore, since in *P. vayredae* the stigmatic papillae

are located in a higher position on the receptive branch (Chapter 2.2), the occurrence and viability of micro-herkogamy were also investigated for the first time in a species with SPP. For this, the variability in the distance between the PP and the stigmatic papillae and its effect on female fitness were evaluated.

## Materials and methods

### *Plant and study area*

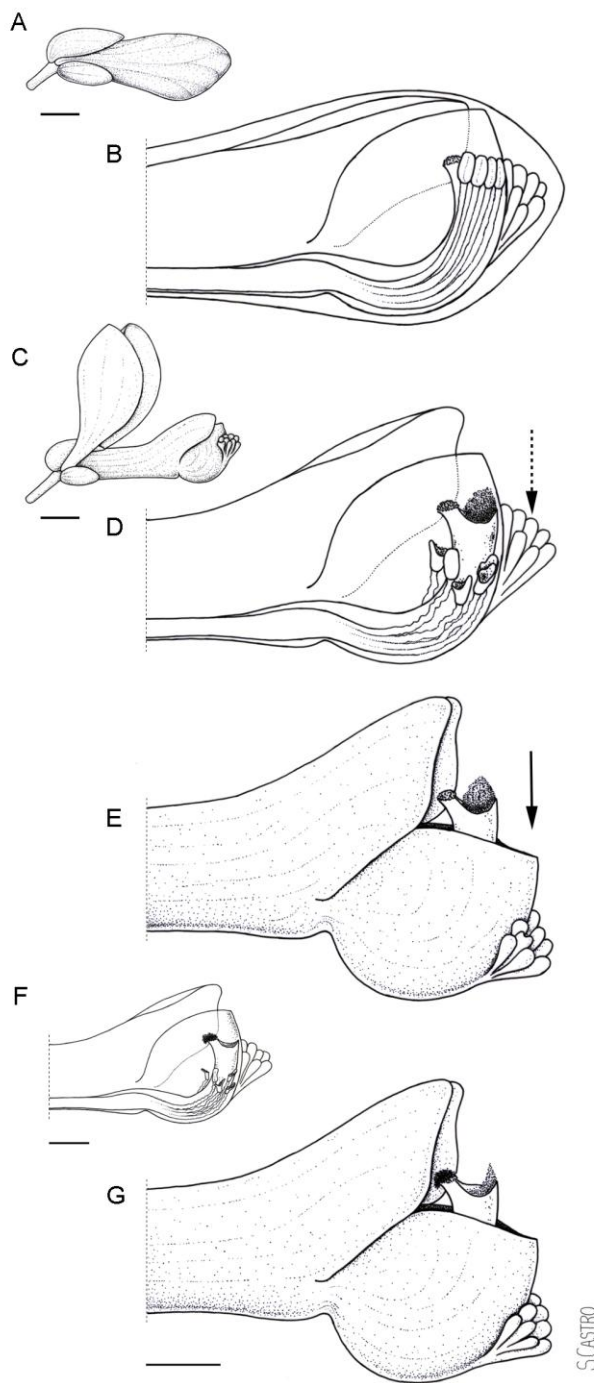
*Polygala vayredae* is an early flowering perennial plant, endemic to the Eastern Pre-Pyrenees (Alta Garrotxa, Girona, Spain), classified as vulnerable by IUCN categories (VV.AA. 2000). This species is entomophilous, with large zygomorphic flowers and a self-incompatibility system at the stigmatic papillae level, depending imperatively on pollinators to set seeds (Chapter 2.1). In the study area the main pollinator was the queens of *Bombus pascuorum* (Apoidea, Hymenoptera) which visits intensively a large number of flowers in a patch and subsequently distances itself from the area (Chapter 4.2).

As a result of the fusion of two monospermic carpels, the stigma is divided into two regions, a sterile one in shape of a basket (the PP) where SPP occurs, and a fertile region where the stigmatic papillae are located (Chapter 2.2; see Fig. 3.1.1). The curved style runs along the corolla tube and fits inside the keel. The anthers open introrsely towards the PP, which is recharged in subsequent pollinator visits through the downward movement of the keel activated by the pressure applied by the insect on the crest (this mechanism is illustrated in Fig. 3.1.2). Despite the deposition of the pollen close to the stigma, no spontaneous self-pollination occurs (Chapter 2.1), and self-interference is mediated by the legitimate pollinator during the flower visit.

The present study was conducted during the spring of 2006 in the Coldecarrera population (Alta Garrotxa, Girona, Spain).

### *Functioning of the PP as self-pollen receptor*

Thirty-five flowers were carefully bagged prior to anthesis. After anthesis, the number of pollen grains deposited in the PP was quantified in the first charge (R1) and in subsequent recharges (R2, R3, R4) until pollen deposition was no longer observed (Fig. 3.1.2). To do this, the movement of the keel made by *B. pascuorum* was simulated by pressing on the crest. The pollen deposited in the PP after each movement of the keel was collected and mounted in 50% glycerine on a microscope slide. Finally, pollen grains were counted under a light microscope.



**Fig. 3.1.2** Schematic representation of the bud, flower and the functioning of the secondary pollen presentation mechanism in the stigmatic pollen presenter of *Polygala vayredae* when flowers are visited by a legitimate pollinator: (A) flower bud; (B) detail of the internal organization in a flower bud with anthers prepared to open introrsely towards the pollen presenter; (C) open flower with androecium and gynoecium enclosed in the corolla; (D) detail of the internal organization of an unvisited open flower with the pollen presenter charged with pollen; (E) first legitimate visit to the flower, where the downward movement of the keel activated by the pressure applied on the crest, exposed the pollen in the pollen presenter and the stigmatic papillae; (F) flower after the visit, returning to the initial state and wilting anthers; (G) flower subjected to a second legitimate visit. Bars = 3 mm for A and C, and 2 mm for the remainder.

#### Efficiency of the PP in pollen dispersion

To evaluate the dispersal of the pollen deposited in the PP, fluorescent powdered dyes were used as pollen analogues (Waser and Price 1982). Although dye and pollen dispersal properties differ (Thomson *et al.* 1986), it has been observed that dye transfer closely resembles pollen transfer when bumblebees, the main pollinators of *P. vayredae*, are the pollen vectors (*e.g.*, Waser 1988; Rademaker *et al.* 1997; Adler and Irwin 2006).

Fluorescent dyes were applied to the PP of 30 newly opened flowers after the self-pollen had been removed. Flowers belonged to several individual plants arranged in clusters of roughly 0.25 m<sup>2</sup>. Three replicates separated by intervals of over 100 m were set within the population. Also, in the intermediate replica a different colored dye was used to avoid erroneous results. After eight days, up to 50 flower samples were collected at several distances from each cluster source (1, 2, 3, 4, 5, 10, 25-50, 50-100 m) and then preserved at -4 °C. In the laboratory, the flowers were examined under UV light using a stereo binocular microscope. The dye powder grains deposited in the stigma were classified into categories by number (class 0 – zero, class 1 – up to 10 grains; class 2 – 11 to 100 grains; class 3 – 101 to 500 grains; class 4 – >500 grains). The proportion of flowers with fluorescent dye was calculated for each distance interval.

#### *SPP, degree of self-interference and female fitness*

To evaluate the influence of SPP on female fitness and on the degree of self-interference the following treatments were applied: 1) open pollinated flowers, *i.e.*, with the possibility of both self- and out-crossed pollen reception; 2) flowers emasculated in the bud and open pollinated, *i.e.*, excluding the possibility of self-pollen reception; and 3) the control group, *i.e.*, bagged and manually self-pollinated flowers. At the onset of fruit development, the corollas (with the style and stigma) were collected and preserved in 70% ethanol to assess the pollen load on the stigmas and the development of pollen tubes through the style. To prevent pollen loss during pistil treatment, pollen loads were evaluated after cutting and squashing the stigmatic papillae on a microscope slide. Styles were then softened with sodium hydroxide 8 N for 4 h, stained with 0.05% aniline blue overnight and squashed in a drop of 50% glycerine (Dafni *et al.* 2005). Samples were observed through an epifluorescence microscope equipped with a UV-2A filter cube (330-380 nm excitation). The number of pollen tubes that successfully developed through the stigmatic papillae and style was recorded. Fruit and seed set were recorded when mature. In these experiments, only the visited flowers were considered. As no spontaneous self-pollination occurs in this species, the presence of pollen on the stigmatic papillae clearly indicates the occurrence of a pollination visitor (for details see Chapters 2.1 and 4.1).

#### *Distance between PP and stigmatic papillae and consequences in the female fitness*

Flower samples were randomly collected throughout the population during the flowering peak and preserved in 70% ethanol. The length of the corolla, stigmatic papillae and PP,

as well as the distance between the latter two structures were measured in 100 flowers. Pollen tube development was evaluated in all flowers following the procedure described above.

### *Statistical analysis*

Differences among the number of pollen grains deposited in the PP after each movement of the keel were evaluated with a Kruskal-Wallis one-way ANOVA on ranks followed by a Tukey test for all multiple pairwise comparisons. Cumulative proportions of the total pollen produced per flower were calculated for each PP recharge with the value of  $5,001 \pm 90.1$  (mean  $\pm$  SE) obtained for this population in Chapter 2.1 being used as a reference.

The proportions of flowers with fluorescent dye among distances (categorical data adjusted to a binomial distribution) were analyzed with a Generalized Linear/Nonlinear Models using a logit link function. The Type 3 Likelihood-Ratio test was computed. The proportion of total delivered pollen was also estimated by inferring the total amount of pollen potentially available in the three clusters (90 flowers with approximately 5,000 pollen grains per flower) and estimating the delivered pollen using the proportion of flowers with fluorescent dye and its amount.

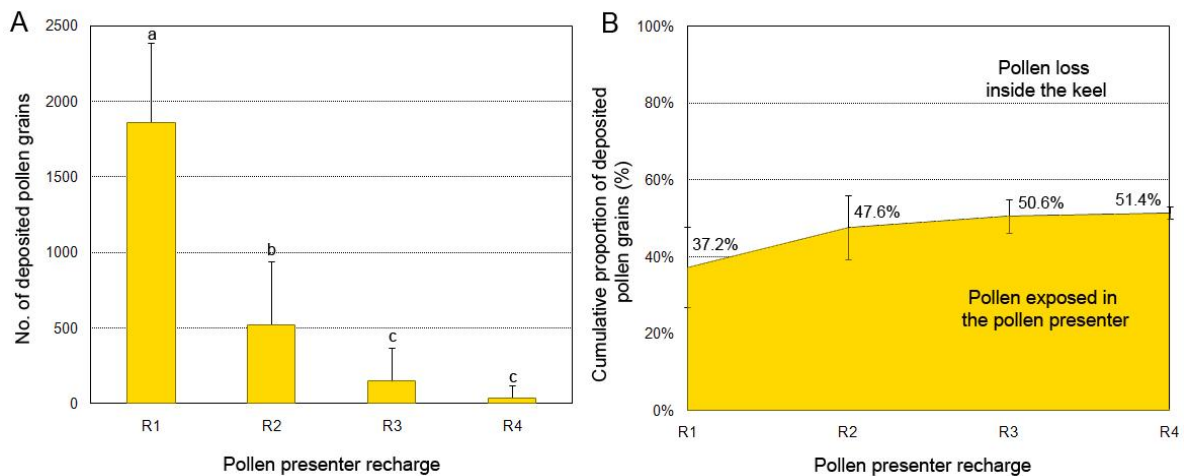
To evaluate the influence of SPP on female fitness, differences among treatments in the number of pollen grains on the stigmas and developed pollen tubes in the styles were analyzed with a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method. The effect of self-interference on fruit and seed production was evaluated with a  $\chi^2$  test for the comparison of more than two proportions and multiple comparison tests for proportions according to Zar (1984).

Spearman's rank order correlation coefficient was calculated to evaluate the relationship between female fitness (number of developed pollen tubes below the stigmatic papillae) and the distance between the PP and stigmatic papillae. As no correlation was found between the PP-stigmatic papillae distance and corolla length ( $R^2 = 0.161$ ,  $P = 0.109$ ), the values were not corrected with this parameter. The mean, standard deviation of the mean (SD) and coefficient of variation (CV) of the PP-stigmatic papillae distance were also calculated.

## Results

### Functioning of the PP as self-pollen receptor

The results of how the PP functions are presented in Figure 3.1.3. Pollen grains were deposited in higher numbers during the first charge of the PP. In subsequent recharges the number of pollen grains deposited on the PP was significantly lower ( $H = 101.03$ ,  $P \leq 0.001$ ; Fig. 3.1.3A). When the results are analyzed in cumulative proportions of the total pollen produced, it was observed that around 48.6% of the pollen grains were lost inside the keel (Fig. 3.1.3B).

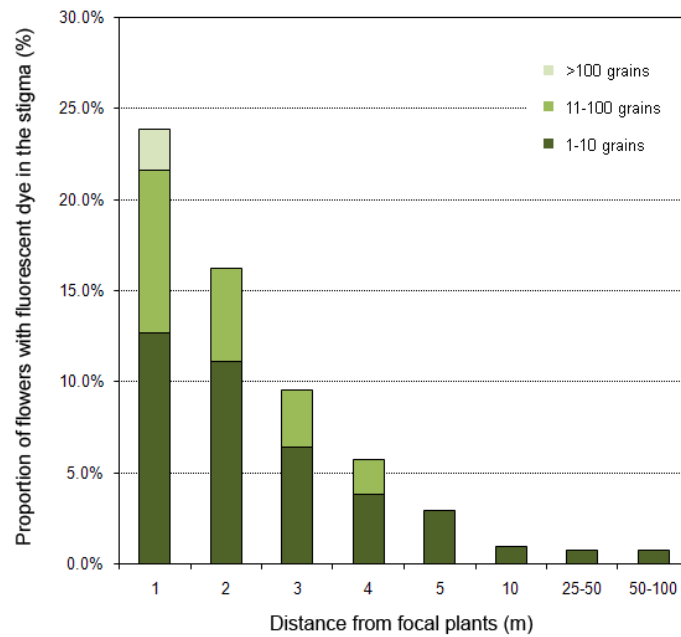


**Fig. 3.1.3** Pollen presenter functioning in *Polygala vayredae*: (A) mean and standard deviation of the mean of the number of pollen grains deposited at the style basket after successive downward movements of the keel (R1, R2, R3 and R4) and (B) estimated cumulative proportion of pollen grains that remain enclosed in the flower after four successive downward movements of the keel.

### Efficiency of the PP in pollen dispersion

Without the pollen presenter pollen exportation is null since the anthers remain enclosed in the keel during the pollinator's visit and are virtually inaccessible to them, with pollen dehiscence occurring inside the keel (Figs. 3.1.2B-C). Thus, the PP allowed the pollen to come into contact with the pollination vector, being able to be exported to the stigmas of other flowers (Fig. 3.1.4). After an exposure of eight days, 16 focal flowers (53.3%) were visited and the fluorescent dye removed. Fluorescent dye was encountered in the stigmatic papillae of 7.8% of the flowers analyzed. It was also observed in small amounts (<10 grains) on the crest, keel and/or petal tips of 3.0% of the flowers analyzed. Although diminished, the pollen flow was found to be higher in the first few meters, dropping significantly as the distance to the focal plants increased ( $\chi^2 = 87.34$ ,  $P < 0.001$ ). A particularly low estimate of the proportion of delivered pollen was obtained (0.44%).





**Fig. 3.1.4** Dispersal of fluorescent dyes among flowers of *Polygala vayredae*: proportions of flowers with fluorescent dye in the stigma represented in terms of the distance to the focal plant cluster. The amount of dye observed in each stigma is shown in categories.

#### *SPP, degree of self-interference and female fitness*

The results of the effect of possible self-interference encouraged by the SPP mechanism on the female fitness of *P. vayredae* are presented in Table 3.1.1. An analysis of the control flowers confirmed the presence of a self-incompatibility system at the stigmatic level, with the near absence of pollen tube development, fruit and seed production. Significant differences for all the variables were found between treatments of the presence/absence of self-pollen). Although they received a significantly higher number of pollen grains (around 117 pollen grains more on average), the flowers with self-interference (not manipulated and with open pollination), developed a significantly lower number of pollen tubes through the style at all levels (Table 3.1.1). Also, in these flowers, the proportions of developed fruits and seeds were significantly lower. The self-pollen that caused the interference was approximately half the amount of the total pollen deposited in the stigmatic papillae during the pollinator's visit.

#### *Distance between PP and stigmatic papillae and consequences in the female fitness*

The distance between the PP and the stigmatic papillae ( $1.24 \pm 0.110$  mm, CV = 8.86%) showed a significant and positive correlation with the number of pollen tubes developed in the style ( $R^2 = 0.274$ ,  $P < 0.01$ ). Thus, female fitness increased slightly as the distance between the PP and the receptive area grew.

**Table 3.1.1.** Results of the treatments carried out to examine the effect of possible self-interference encouraged by the secondary pollen presentation mechanism on female fitness of *Polygala vayredae*.

Treatment	n	No. pollen grains	No. pollen tubes along the style			No. fruits (fruit set)	No. seeds (seed ovule ratio)
			Beginning	Middle	End		
With self-interference	86	230 ± 16.3 <sup>a</sup>	5.3 ± 0.56 <sup>a</sup>	4.8 ± 0.54 <sup>a</sup>	4.1 ± 0.48 <sup>a</sup>	56 (65.1%) <sup>a</sup>	84 (48.8%) <sup>a</sup>
Without self-interference	30	113 ± 18.3 <sup>b</sup>	8.9 ± 0.91 <sup>b</sup>	8.1 ± 0.85 <sup>b</sup>	6.9 ± 0.77 <sup>b</sup>	26 (86.7%) <sup>b</sup>	39 (65.0%) <sup>b</sup>
Control	35	29 ± 3.5 <sup>c</sup>	0.6 ± 0.17 <sup>c</sup>	0.4 ± 0.15 <sup>c</sup>	0.3 ± 0.15 <sup>c</sup>	2 (5.7%) <sup>c</sup>	3 (4.3%) <sup>c</sup>
Comparison test		$H = 72.59^{***}$	$H = 46.79^{***}$	$H = 47.61^{***}$	$H = 45.71^{***}$	$\chi^2 = 50.17^{***}$	$\chi^2 = 80.11^{***}$

Notes: Values are given as means and standard errors of the mean. Differences in the number of pollen grains and tubes among treatments were evaluated with a Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method; differences in the fruit and seed production among treatments were evaluated with a  $\chi^2$  test for the comparison of more than two proportions and multiple comparison tests for proportions following Zar (1984). \*\*\* $P \leq 0.001$ .

## Discussion

In outcrossing species, a conflict often arises when deciding between selecting to present pollen and stigmas in similar positions to improve pollination success and selecting to keep them apart to minimize or avoid interference between pollen exportation and pollen receipt on the stigma (Lloyd and Webb 1986; Webb and Lloyd 1986). Secondary pollen presentation, which evolved in several groups of Angiosperms, allows the pollen and stigmas to be presented in similar positions within a blossom (Carolin 1960; Yeo 1993; Ladd 1994). Nevertheless, even though this is a widespread, highly diversified and morphologically studied feature (for a review see Yeo 1993), the functional aspects and consequences of such a mechanism on plant fitness are still largely unknown. The present study has evaluated, for the first time, the consequences of SPP in both pollen exportation and pollen reception, in addition to the possible detrimental effects of the proximity of the self-pollen and stigmatic area.

Flowering plants that rely on animal vectors to transport the pollen grains to conspecific stigmas of other flowers are exposed to great uncertainty and are frequently subjected to high rates of pollen loss (e.g., Inouye *et al.* 1994; Morris *et al.* 1994). Several different pollen fates may occur in this pathway, ranging from non-exportable pollen to pollen loss in their own stigmas during removal and transport, or at the presentation site (Inouye *et al.* 1994; Harder and Wilson 1998). In *P. vayredae* the SPP mechanism presents considerable intrinsic pollen losses. The flowers produced roughly 5000 pollen grains, but only around 51% became available on the PP for exportation during the flower's lifespan. The remaining pollen (non-exportable pollen) dwelled inside the corolla without the

possibility of being exposed and dispersed. This pollen loss due to the SPP mechanism reduced the opportunities for mating by half, even before the pollen had the chance to be presented to the vector. Despite the fact that this precollection pollen loss (following Inouye *et al.* 1994) is generally overlooked in species with primary pollen presentation (but see Rademaker *et al.* 1997), in species with SPP, if the objective is to explore the efficiency of the mechanism, this parameter must be carefully analyzed, once it becomes part of an additional step during the traveling schedule of the pollen grain (*i.e.*, secondary relocation of the pollen). Consequently, more studies on precollection pollen losses involving other species are needed for a more in-depth evaluation of the SPP mechanism.

In *P. vayredae* the downward movement of the keel activated by a legitimate visitor with some weight, such as the long-tongued bumblebee, exposes the PP and allows for the dispersal of pollen among the flowers. The results of this study show that in the Coldecarrera population the pollen flow and the proportion of pollen received were low in 2006. This was due to pollinator limitation (Chapters 2.1 and 4.2) and, in visited focal flowers, to high pollen losses by the pollen vector during pickup, transport and delivery. Nonetheless, despite the intrinsic losses of the mechanism, pollinator limitation (both cases of precollection pollen losses) and subsequent pollen losses during transport on the pollen vector (predeposition pollen loss), the pollen transport efficiency observed was similar to what has been reported for species with similar granular pollen (percentage of removed pollen delivered below 0.5%; Thomson and Thomson 1989; Galen 1992; Rademaker *et al.* 1997). On the other hand, if only the pollen available for transportation is considered, pollen transfer efficiency was slightly higher than what was observed in species with primary pollen presentation (*e.g.*, Harder and Thomson 1989; Thomson and Thomson 1989; Galen 1992; Rademaker *et al.* 1997). An improvement in male fitness through SPP has been suggested in other species of *Polygala*, where precise depositions of the pollen and flower asymmetry lead to sexual isolation (Brantjes 1982).

Self-interference has been described as the conflict between male and female functions due to their close proximity, which despite improving the pollen delivery/reception, can also lead to detrimental effects on plant fitness (Cesaro *et al.* 2004). In *P. vayredae* we observed that the presence of self-pollen in the stigmatic papillae reduced the number of developed pollen tubes as well as the number of fruits and seeds owing to interference with outcross pollen, although a self-incompatibility system clearly limits its negative effects. As suggested by Webb and Lloyd (1986) and recently shown empirically by Koelling and Karoly (2007), the presence of a self-incompatibility system may minimize or eliminate this conflict of interests. Additionally, for the same

purpose, the temporal separation of male and female functions was also proposed (Lloyd and Webb 1986) and evaluated (Lloyd and Yates 1982; Routley and Husband 2006). In the few available studies involving species with SPP, similar features appear to be present. For example, in *Cephalanthus occidentalis* L. a higher growth rate of outcrossed pollen tubes and an inhibition of self-pollen tubes at the base of the style largely prevented selfing (Imbert and Richards 1993), and in *Rauvolfia grandiflora* Mart. ex A. DC. a late acting self-incompatibility mechanism was also observed (Lopes and Machado 1999). In several species of *Campanula* L., on the other hand, the tactile stimulation of the style hairs, where SPP occurs, resulted in the control of male and female phases, reducing the maturation of the former while accelerating that of the latter (Nyman 1993). In *P. vayredae* a mechanism of self-incompatibility prevents self-fertilization with rejection occurring at the stigmatic papillae level (results herein and Chapter 2.1). This mechanism will not avoid self-interference but could prevent its potential negative effects by primarily increasing maternal fitness (Lloyd and Webb 1986).

As odd as it may seem, considering the major advantages proposed for SPP, micro-herkogamy has also been suggested as another mechanism that may emerge to avoid self-interference (Ladd 1994; Lopes and Machado 1999). In *P. vayredae* it was previously observed that stigmatic papillae are located somewhat in a higher position on their stigmatic branch in relation to the PP (Chapter 2.2). In this study, the relationship between the PP-stigmatic papillae distance and female fitness revealed a slight increase in fitness with increased distance. Thus, this micro-separation appears to be slightly advantageous for plant fitness. In *Narcissus assoanus* Dufour, even though SPP is not present, it was experimentally observed that floral traits like herkogamy limited the cost of self-interference, which exerted a detrimental effect on seed set (Cesaro *et al.* 2004). As self-pollination can reduce opportunities for outcrossing, selection will favor floral traits that reduce self-interference and improve outcrossing (Webb and Lloyd 1986). This could be of special importance in xenogamous species with SPP mechanisms, where self-interference has significant detrimental consequences.

This is the first study that has evaluated experimentally the consequences of SPP in male and female fitness. The study also examines the occurrence of herkogamy in a species with SPP and its effects on female fitness. It was observed that the SPP mechanism could be advantageous in the accurate delivery/reception of pollen, but it may also have its costs. The loss of male gametes (an intrinsic loss of the device) and/or the self-interference (a consequence of the device) must be taken into account overall. Micro-herkogamy appeared to be advantageous as it led to a slight increase in female fitness.

However, further studies are needed on several taxonomic groups in order to understand the evolution of SPP. Due to the high diversity in SPP structures, Polygalaceae would appear to be a very interesting family to be used for such purposes as well as for evaluating differences between distinct mechanisms/morphologies, providing new insights in the real adaptive significance of SPP.

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## Chapter 4

### Pollination ecology

#### 4.1 Floral traits variation, legitimate pollination and nectar robbing in *Polygala vayredae* (Polygalaceae)

Chapter section submitted as an original article to SCI journal:

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## Abstract

The variation on floral morphology and its effect on flower visitors of *Polygala vayredae* Costa (Polygalaceae), a narrow endemic species from Oriental pre-Pyrenees, were examined. First, to account with the main floral reward (*i.e.*, nectar), the relationship between dimension of nectar gland and nectar production was investigated. Second, floral traits variation was assessed within and between the three most representative populations of the species. Finally, the role of several floral traits in the female fitness was evaluated. Furthermore, as nectar robbing was highly frequent, preferences of robbers for specific floral traits and their impacts on legitimate pollinations were also evaluated. The flowers of this species are characterized by significant variation in floral characters and nectar rewards. A significant and positive correlation between nectar gland dimension and nectar production per flower was observed, with gland dimension being a good measure to infer the rewards offered by the flowers of *P. vayredae*. In general, corolla traits were significant and positively correlated with each other. Nectar revealed to be an important trait in flower-visitor interactions, with legitimate pollinations being primarily influenced by this floral reward. Negative correlations between robbing frequency and legitimate pollinations were observed in two of the studied populations, and positive correlations between flower size and robbing frequency were observed in one population. An indirect negative selection over phenotypic floral traits mediated by nectar robbers is proposed. Overall, the general papilionate floral morphology, despite being highly elaborated, did not influence significantly the behaviour of the pollinators.

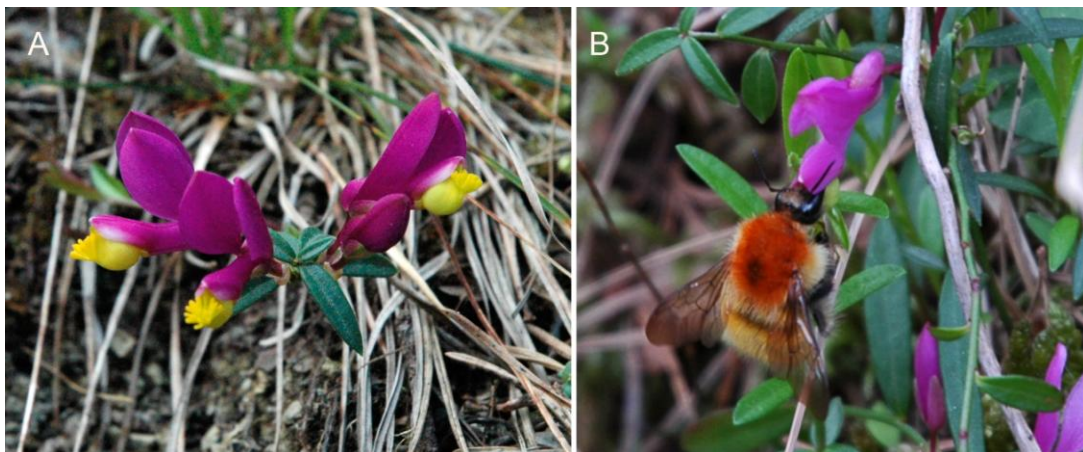
**Keywords** floral morphology; endemic species; nectar rewards; nectar robber; phenotypic selection

## Introduction

In animal-pollinated plants, floral morphology has tightly evolved with its pollinators (Stebbins 1970; Galen 1999). Thus, it is possible to interpret several floral traits as adaptations to certain pollination vectors that result from effective selective pressures exerted by them over floral morphology. For this, it is essential to have phenotypic and genetic variation in the floral traits directly involved with pollinator attraction and efficiency, which can result in different reproductive outputs and further selection (Herrera 1990, 1996). Facing a variable assemblage of floral displays, pollinators have shown to prefer plants with certain floral traits, such as, high number of flowers (Mitchell *et al.* 2004; Benitez-Vieyra *et al.* 2006), large flowers (Ishii and Harder 2006; Celedón-Neghme *et al.* 2007), large nectar rewards (Thomson 1986; Fenster *et al.* 2006), specific corolla colour or shape (Sutherland and Vickery 1993; Gómez *et al.* 2006) or particular flowering time (Hall and Willis 2006).

Nevertheless, recent works have shown that the selection of flower traits not only results from pollinator-mediated selection, but is rather a dynamic process where other biotic and abiotic factors can also exert strong selective pressures (*e.g.*, Herrera 1993; Herrera *et al.* 2002; Gómez 2003; Giles *et al.* 2006; Sánchez-Lafuente 2007). Among the biotic factors, nectar robbing was shown to be an important feature affecting the plant fitness (*e.g.*, Maloof and Inouye 2000; Irwin *et al.* 2001). Understanding the effects of nectar robbers on the visited plants and on the remaining floral visitors is especially important considering the high rates of robbing and the high proportion of robber's visitation observed in several species (Maloof and Inouye 2000). In some of these works, a diverse array of effects of nectar robbing on plant reproductive success, have already been observed. Negative interactions of nectar robbers, as changes in the patterns of available nectar, reduction of flower attractiveness and/or damaging of floral structures, have been frequently described to affect further floral interactions, visitor's behaviour and plant fitness in several species (*e.g.*, Zimmerman and Cook 1985; Irwin and Brody 1999; Traveset *et al.* 1998; Zhang *et al.* 2007). On the other hand, positive interactions, such as the accidental involvement of nectar robbers in pollination when accessing for nectar (*e.g.*, Higashi *et al.* 1988; Navarro *et al.* 1993, 2000), and neutral interactions (*e.g.*, Arizmendi *et al.* 1996; Morris 1996), have also been observed. In the particular case of tubular flowers nectar robbing may act as an opposite selective force that balances the selection of floral traits by specialist pollinators (Lara and Ornelas 2001). Nonetheless, to date, only a few studies have shown the direct or indirect effect of nectar robbers on the selection of plant traits (but see Irwin 2006).

The elaborated flowers morphology of Polygalaceae (keel flowers) and its superficial resemblance with Fabaceae raise questions on the function of floral components and its role in pollinator's attraction (Eriksen 1993; Westerkamp 1997, 1999). Keel flowers are generally described as bee pollinated (Faegri and van der Pijl 1979; Westerkamp 1997) and are composed by two elementary functional structures: a flag for visual advertisement/attraction, and a keel protecting the sexual organs (Westerkamp 1997). In *Polygala* L. (and in Polygalaceae in general), the two lateral petaloid sepals are described as the advertisement structure, while the keel, besides protecting the organs, presents an appendage (crest) for visitor's landing (Fig. 4.1.1A). Furthermore, the fusion and imbrication of the upper petals produce a tubular-like flower that can influence visitor's assemblage, including nectar robbers (Navarro 2001; Lara and Ornelas 2001). Despite of these observations, in Polygalaceae, no studies have been developed to evaluate the direct correlation between floral traits and legitimate pollination, and its consequences on floral traits selection.



**Fig. 4.1.1** *Polygala vayredae*: (A) flowers and (B) queen of *Bombus pascuorum* visiting the flowers.

The present work focuses and explores how the quantitative variation in floral traits influences the interactions between the flowers of *Polygala vayredae* Costa and its visitors. *P. vayredae* is a narrow endemic species from Oriental pre-Pyrenees and an entomophilous plant that strictly relies on pollen vectors to set fruit (Chapter 2.1). The shape of its flowers requires the visit of specialized pollinators, such as long-tongued insects (namely queens of *Bombus pascuorum* and *Anthophora* sp.), which during nectar exploitation move downwards the keel and get in contact with the concealed reproductive organs, accomplishing pollination (Fig. 4.1.1B). Furthermore, nectar robbing has been described as a steady feature in this species, with high robbing frequencies and high visitation rates being observed in its main populations (Chapter 4.2). Our objectives were

to investigate the variation in floral characters within and among populations, to assess how the variation in floral traits influences legitimate pollinations, and to evaluate the extent of nectar robbing as a factor of selective pressures. For this, morphometric analyses of floral characters were performed on flowers from the three main populations of *P. vayredae*, with the data on nectar reward and nectar robbing being included in the analysis. Standard regression methods were applied for detecting patterns of natural selection on floral traits.

## Materials and methods

### *Plant and study area*

*Polygala vayredae* is an early flowering, perennial and self-incompatible species, endemic from Oriental pre-Pyrenees, where it occurs in a few dense populations distributed in a restricted area of approximately 12 km<sup>2</sup> in Alta Garrotxa, Girona (Catalunya, Spain). This species presents large zygomorphic flowers, arranged in small axilar inflorescences of 1-3 flowers. The two lateral petaloid sepals and the corolla tube are pink, while the keel, where androecia and gynoecia are concealed, is sulphur yellow (Fig. 4.1.1A). Flowers present a large life span and nectar rewards are produced in a gland at the base of the corolla tube (Chapters 2.1 and 2.3). *P. vayredae* strictly relies on pollinators to set fruit and is frequently subjected to pollen/pollinator limitation and nectar robbing (Chapters 2.1 and 4.2).

The study was carried during spring of 2005 by collecting flowers from distinct individuals in three populations: Montmajor (n = 131), Serrat dels Boixos (n = 76) and Coldecarrera (n = 112). Flower samples were preserved in ethanol 70% until laboratory analysis. Information about the main floral visitors and frequency of nectar robbing for each population is given in Table 4.1.1 (see also Fig. 1.1).

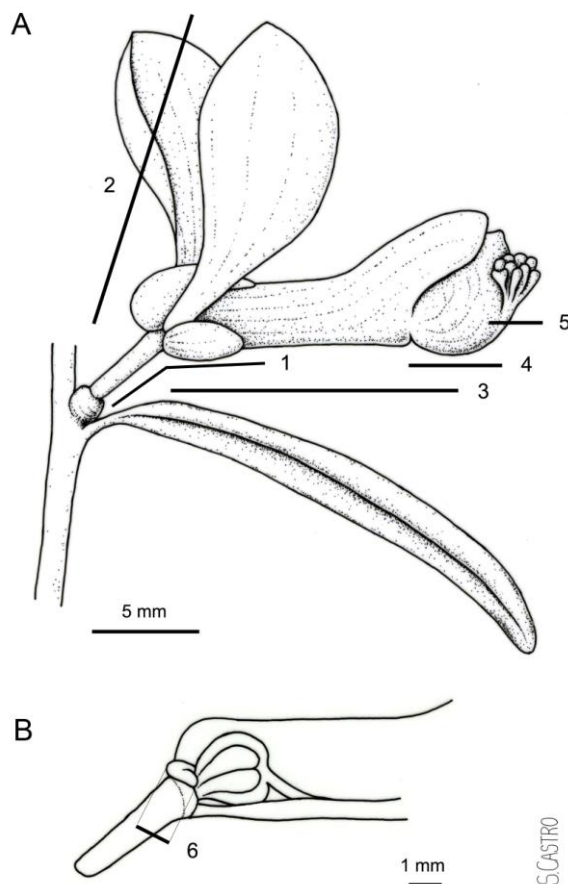
**Table 4.1.1** Studied populations of *Polygala vayredae*.

Populations	Habitat	Altitude (m)	Main pollinators	Main nectar robbers	Nectar robbing (%)
Montmajor	Rocky place ( <i>Saxifragion mediae</i> )	1,070	<i>B. pascuorum</i>	<i>Macroglossum stellatarum</i> <i>B. terrestris</i>	94.9
Serrat dels Boixos	Mesophytic meadow ( <i>Mesobromion</i> , <i>Brometalia erecti</i> )	750	<i>B. pascuorum</i> <i>Anthophora</i> sp.	<i>B. terrestris</i> <i>Gonepteryx rhamni</i>	92.1
Coldecarrera		630	<i>B. pascuorum</i> <i>Anthophora</i> sp.	<i>B. terrestris</i> <i>B. pratorum</i>	92.1

Notes: Nectar robbing was assessed by observing up to 1,000 randomly selected flowers from distinct plants in 2005.

### *Relationship between nectar gland and nectar production*

To include a measure of the general offered reward, the correlation between nectar gland dimension and nectar production was assessed. For this, nectar production was quantified in 30 flowers with 1-2 days-old, randomly selected along Coldecarrera population. Flowers of this age were selected because the production of nectar in this species occurs mainly during the first three days of the flower lifespan (Chapter 2.1). The flowers were identified and bagged. In each flower, after 24 and 48 h, nectar was extracted and quantified with a capillary micropipette, and sugar concentration was determined (in w/w, %) with a portable refractometer. The amount of sugar produced by each flower was calculated according with Prys-Jones and Corbet (1987). After nectar quantification, flowers were collected for morphometric analysis of the nectar gland (Fig. 4.1.2).



**Fig. 4.1.2** Illustration of *Polygala vayredae*: (A) complete flower and (B) detail on the location of the nectar gland. Morphometric measures under study: (1) angle of flower presentation, and (2) length of wings, (3) corolla tube, (4) keel, (5) crest and (6) nectar gland.

### *Variation on floral morphology and its effect on flower visitors*

Morphometric analyses were performed on *P. vayredae* flowers by collecting the following measurements: angle of flower presentation (directly involved with flower presentation to visitors), length of the wings (involved in advertisement), length of the corolla tube (a limiting factor in nectar access), length of the keel (involved in advertisement and organ

protection), length of the crest (a foot holding for visitors) and length of the nectar gland (an indirect measurement of floral reward) (Fig. 4.1.2). Measurements were taken on photographs using analySIS 5.0 software. Furthermore, in each flower, signs of nectar robbing were also recorded. Nectar robbing (*sensu* Inouye 1980) was easily evaluated due to the visible and repeated incisions made in the corolla by robbers, and allowed an approximation to the nectar robbing intensity. In some extent, this measure could be underestimated, as nectar robbers may use the existing holes, and behave as secondary nectar robbers. Nonetheless, it should be noticed that the frequency of interactions obtained for the main nectar robber (*B. terrestris*) during the year of study (Chapter 4.2) was consistent with the robbing intensity observed in the present work. Pistils were treated with sodium hydroxide 8 N for 4 h, stained overnight with aniline blue 0.05% and squashed in a drop of glycerine 50% (Dafni *et al.* 2005). Finally, they were observed through an epifluorescence microscope with a UV-2A filter cube (330-380 nm excitation). The number of pollen tubes able to germinate and grow throughout the stigmatic papillae and style was recorded. As this species is self-incompatible, blocking the pollen tube development mainly at the stigmatic papillae (Chapter 2.1), the presence/absence and number of pollen tubes in the style were used as measures of legitimate visit and successful pollination.

### *Statistical analysis*

The relationship between nectar gland dimension and nectar production (nectar volume and amount of sugar) was assessed by applying the Spearman's rank order correlation coefficients.

Descriptive statistics of flower traits and nectar robbing intensity (*i.e.*, number of holes in the corolla) were calculated for each population. Variation in these traits within and among the populations was investigated with one-way ANOVA, followed by a Tukey or Dunn's test for pairwise multiple comparisons. For each studied population, relationships among morphometric variables were investigated through the Spearman's rank order correlation coefficients. Relative variability of each morphometric character was assessed by the coefficient of variation.

Descriptive statistics of each flower trait and nectar robbing intensity were also calculated for successful (with pollen tube development below stigmatic papillae) and unsuccessful flowers (without pollen tube development below stigmatic papillae). Variation in floral morphology and robbing intensity between these two groups was investigated with a *t* test.



For each population, linear multiple regression analyses were applied for detecting the relationship between the occurrence of legitimate pollinations (given by the number of developed pollen tubes) and morphometric characters. These approaches allowed, in some extent, to perceive pollinator's preferences and to investigate directional selection of floral traits (Lande and Arnold 1983). Correlated variables (corolla tube, keel, crest and wings) were integrated using principal component analysis (PCA). The two first factors explained more than 80% of the variability and were both used in the regression analysis. The intensity of nectar robbing was also included. The same procedures were applied for detecting the relationship between the occurrence of nectar robbing and morphometric characters. In any of the regression analysis, tolerance was higher than 0.75 and the variance inflated factors were below than 1.35.

## Results

### *Relationship between nectar gland and nectar production*

The nectar gland dimension was significantly and positively correlated with both nectar volume ( $R^2 = 0.688$ ,  $P < 0.001$ ) and amount of sugar ( $R^2 = 0.716$ ,  $P < 0.001$ ) produced per flower. Thus, the flowers of *P. vayredae* with bigger nectar glands produced higher nectar rewards.

### *Flower morphology*

The results of the morphometric analysis for each floral trait are described in Table 4.1.2. Multivariate analysis of variance revealed significant differences among populations ( $F = 6.86$ ,  $P < 0.001$ ). With exception of the nectar gland, the mean values for each floral character varied significantly among populations, with the Montmajor individuals presenting slightly larger flowers (Table 4.1.2). Coldecarrera was the most heterogeneous population, showing the highest values of coefficient of variation (CV) for all the parameters and the higher intensity of nectar robbing. The corolla tube and the keel were the less variable characters (overall CV of 5.2% and 6.6%, respectively), while the nectar gland and the crest were the most heterogeneous (overall CV of 13.9% and 23.2%, respectively).

**Table 4.1.2** Intensity of nectar robbing and mean, standard deviation of the mean and coefficient of variation (given in parenthesis; %) of the floral characters studied in three populations of *Polygala vayredae*.

Floral traits Populations	Robbing intensity	Angle (°)	Corolla tube (mm)	Keel (mm)	Crest (mm)	Wing (mm)	Nectar gland (mm)
Montmajor	1.3 ± 0.85 <sup>a</sup>	152.8 ± 10.97 <sup>a</sup> (7.2)	14.8 ± 0.74 <sup>a</sup> (5.0)	6.6 ± 0.38 <sup>a</sup> (5.8)	2.4 ± 0.45 <sup>a</sup> (18.8)	16.0 ± 1.02 <sup>a</sup> (6.4)	0.88 ± 0.130 (14.8)
Serrat dels Boixos	1.0 ± 0.73 <sup>b</sup>	154.5 ± 11.64 <sup>a</sup> (7.5)	14.6 ± 0.73 <sup>a,b</sup> (5.0)	6.3 ± 0.37 <sup>b</sup> (5.9)	2.1 ± 0.46 <sup>b</sup> (21.9)	15.4 ± 1.10 <sup>b</sup> (7.1)	0.89 ± 0.102 (11.5)
Coldecarrera	1.7 ± 0.88 <sup>c</sup>	149.1 ± 12.34 <sup>b</sup> (8.3)	14.5 ± 0.78 <sup>b</sup> (5.4)	6.3 ± 0.45 <sup>b</sup> (7.1)	2.1 ± 0.52 <sup>b</sup> (24.8)	16.1 ± 1.26 <sup>a</sup> (7.8)	0.89 ± 0.132 (14.8)
Comparison test	$H =$ 59.54***	$F = 5.46^{**}$	$F = 3.61^{*}$	$H =$ 24.48***	$F =$ 18.97***	$H = 12.26^{**}$	$F = 0.18$ <i>n.s.</i>
All populations		151.9 ± 11.79	14.6 ± 0.76	6.4 ± 0.42	2.2 ± 0.51	15.9 ± 1.15	0.89 ± 0.124
Range		111.7 - 191.0	11.6 - 16.8	5.4 - 7.7	1.1 - 3.8	12.8 - 19.2	0.48 - 1.32
CV (%)		7.8	5.2	6.6	23.2	7.2	13.9

Notes: Robbing intensity is given as mean and standard deviation of the mean of the number of holes in the corolla.

Different letters reveals significant differences. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; *n.s.* - not significant.

Correlations between floral characters within each population are presented in Table 4.1.3. In general, the corolla traits, *i.e.*, corolla tube, keel, crest and wing, were significantly and positively correlated with each other. The exceptions were the crest vs. wing in Serrat dels Boixos population, and the crest vs. corolla tube in Coldecarrera population, both with low positive and not significant correlation coefficients. The relationships between the nectar gland and corolla traits were only significantly (and positively) correlated for the corolla tube and the wing in Montmajor population, being not significantly correlated for the remaining traits in the other populations. No correlations were expected between the angle of flower presentation and the remaining variables, but significant and positive correlations were found between this floral trait and the nectar gland in Montmajor and Coldecarrera populations.

**Table 4.1.3** Spearman correlation coefficients between all the analyzed floral characters for each studied population.

Population	Floral traits	Angle	Corolla tube	Keel	Crest	Wing
Montmajor	Corolla tube	0.171 <i>n.s.</i>	-	-	-	-
	Keel	0.065 <i>n.s.</i>	<b>0.442***</b>	-	-	-
	Crest	0.007 <i>n.s.</i>	<b>0.228**</b>	<b>0.685***</b>	-	-
	Wing	-0.120 <i>n.s.</i>	<b>0.584***</b>	<b>0.462***</b>	<b>0.415***</b>	-
	Nectar gland	<b>0.219*</b>	<b>0.394***</b>	0.125 <i>n.s.</i>	-0.014 <i>n.s.</i>	<b>0.339***</b>
Serrat dels Boixos	Corolla tube	0.171 <i>n.s.</i>	-	-	-	-
	Keel	-0.016 <i>n.s.</i>	<b>0.434***</b>	-	-	-
	Crest	0.042 <i>n.s.</i>	<b>0.245*</b>	<b>0.530***</b>	-	-
	Wing	-0.112 <i>n.s.</i>	<b>0.600***</b>	<b>0.511***</b>	0.178 <i>n.s.</i>	-
	Nectar gland	0.110 <i>n.s.</i>	0.104 <i>n.s.</i>	0.148 <i>n.s.</i>	-0.026 <i>n.s.</i>	0.154 <i>n.s.</i>
Coldecarrera	Corolla tube	0.025 <i>n.s.</i>	-	-	-	-
	Keel	-0.118 <i>n.s.</i>	<b>0.297**</b>	-	-	-
	Crest	-0.181 <i>n.s.</i>	0.056 <i>n.s.</i>	<b>0.635***</b>	-	-
	Wing	-0.065 <i>n.s.</i>	<b>0.491***</b>	<b>0.509***</b>	<b>0.349***</b>	-
	Nectar gland	<b>0.317***</b>	0.141 <i>n.s.</i>	0.041 <i>n.s.</i>	0.022 <i>n.s.</i>	0.076 <i>n.s.</i>

Notes: Significant coefficients are highlighted in bold. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; *n.s.* - not significant.

#### *Variation on floral morphology and its effect on flower visitors*

*Polygala vayredae* flowers received visits from legitimate pollinators and/or nectar robbers. From the 319 flowers collected in the three populations under study, 153 (48.0%) were successfully pollinated, while the remaining 166 (52.0%) did not receive any efficient visit. Floral traits for successful and unsuccessful flowers are described in Table 4.1.4. Successful flowers presented significantly smaller keels ( $t = 2.88$ ,  $P < 0.01$ ) and crests ( $t = 3.82$ ,  $P < 0.001$ ), and significantly larger nectar glands ( $t = -3.33$ ,  $P < 0.001$ ). Furthermore, the intensity of nectar robbing was significantly lower in successful flowers in comparison with unsuccessful ones ( $t = 4.60$ ,  $P < 0.001$ ). No significant differences were found between successful and unsuccessful flowers in the remaining characters (Table 4.1.4).

**Table 4.1.4** Intensity of nectar robbing and mean and standard deviation of the mean of floral traits for successful and unsuccessful flowers of *Polygala vayredae*.

Floral traits	n	Robbing intensity	Angle (°)	Corolla tube (mm)	Keel (mm)	Crest (mm)	Wing (mm)	Nectar gland (mm)
Successful flowers	153	1.1 ± 0.67	151.4 ± 11.87	14.6 ± 0.83	6.4 ± 0.43	2.1 ± 0.50	15.9 ± 1.27	0.91 ± 0.130
Unsuccessful flowers	166	2.0 ± 0.88	152.4 ± 11.73	14.7 ± 0.69	6.5 ± .40	2.4 ± 0.49	15.9 ± 1.03	0.86 ± 0.115
<i>t</i> test		<i>t</i> = 4.60***	<i>t</i> = 0.76 <i>n.s.</i>	<i>t</i> = 1.47 <i>n.s.</i>	<i>t</i> = 2.88**	<i>t</i> = 3.82***	<i>t</i> = 0.15 <i>n.s.</i>	<i>t</i> = -3.33***

Notes: Successful flowers refer to an efficient pollination performed by a legitimate pollinator. As this species is self-incompatible blocking the development of self pollen tubes at the stigmatic papillae, successful and unsuccessful flowers were easily determined by the presence or absence of pollen tubes in the style. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; *n.s.* - not significant.

The results of the multiple linear regression analysis of the effect of floral traits in legitimate pollinations are presented in Table 4.1.5. Several differences among the studied populations were found on the contribution of each character. In Montmajor and Coldecarrera populations the variables nectar gland and nectar robbing intensity were significantly correlated with the occurrence of an efficient visit from a legitimate pollinator. In both populations, while the nectar gland was positively correlated and presented a high contribution when explaining successful pollinations (regression coefficients of 7.2 and 14.0), the nectar robbing intensity was negatively correlated, (regression coefficients of -1.5 and -3.9; Table 4.1.5). The remaining variables were not significantly correlated and presented low regression coefficients. In Serrat dels Boixos a different scenario was observed, with the factors 1 and 2 of the PCA being the only significant variables presenting opposite correlations (Table 4.1.5). Nevertheless, one should note that despite being not significant, the nectar gland presents a highly positive regression coefficient (10.4; Table 4.1.5).

**Table 4.1.5** Results of multiple linear regressions to evaluate the effect of the floral traits and nectar robbing intensity on the probability of successful pollination in each of the studied populations.

Floral traits	Montmajor				Serrat dels Boixos				Coldecarrera			
	Coef.	$\beta$	<i>t</i>	<i>P</i>	Coef.	$\beta$	<i>t</i>	<i>P</i>	Coef.	$\beta$	<i>t</i>	<i>P</i>
Angle	0.015	0.055	0.662	0.509	-0.039	-0.072	-0.665	0.508	0.026	0.045	0.465	0.643
PCA factor 1	0.189	0.099	1.136	0.258	<b>1.422</b>	<b>0.344</b>	<b>3.179</b>	<b>0.002</b>	-0.264	-0.054	-0.586	0.559
PCA factor 2	0.261	0.081	0.898	0.371	<b>-1.439</b>	<b>-0.222</b>	<b>-2.066</b>	<b>0.042</b>	-0.345	-0.050	-0.542	0.589
Nectar gland	<b>7.192</b>	<b>0.311</b>	<b>3.293</b>	<b>0.001</b>	10.429	0.169	1.559	0.123	<b>13.955</b>	<b>0.255</b>	<b>2.573</b>	<b>0.011</b>
Robbing intensity	<b>-1.533</b>	<b>-0.225</b>	<b>-2.667</b>	<b>0.009</b>	-0.209	-0.011	-0.103	0.919	<b>-3.944</b>	<b>-0.188</b>	<b>-1.988</b>	<b>0.049</b>

Notes: Coef. - regression coefficient;  $\beta$ , regression coefficient with standardize variables to a mean of 0 and a standard deviation of 1. The coefficients with *P* < 0.05 are highlighted in bold.

**Table 4.1.6** Results of multiple linear regressions to evaluate the effect of the floral traits on the probability of receive visit(s) by a nectar robber in each of the studied populations.

Floral traits	Montmajor				Serrat dels Boixos				Coldecarrera			
	Coef.	$\beta$	<i>t</i>	<i>P</i>	Coef.	$\beta$	<i>t</i>	<i>P</i>	Coef.	$\beta$	<i>t</i>	<i>P</i>
Angle	0.011	0.176	1.755	0.082	-0.004	-0.080	-0.686	0.495	-0.006	-0.073	-0.847	0.399
PCA factor 1	-0.045	-0.084	-0.883	0.379	0.094	-0.219	-1.885	0.064	-0.070	-0.127	-1.400	0.164
PCA factor 2	-0.107	-0.142	-1.500	0.137	-0.007	-0.011	-0.094	0.925	<b>0.251</b>	<b>0.270</b>	<b>2.921</b>	<b>0.004</b>
Nectar gland	-0.814	-0.137	-1.376	0.172	-0.332	-0.052	-0.446	0.657	-0.335	-0.050	-0.509	0.612

Notes: Coef. - regression coefficient;  $\beta$ , regression coefficient with standardize variables to a mean of 0 and a standard deviation of 1. The coefficients with *P* < 0.05 are highlighted in bold.

Nectar robbing was a steady feature in all the studied populations. The results of the multiple linear regression analysis of the effect of floral traits in the occurrence of nectar robbing are presented in Table 4.1.6. Positive and significant correlation coefficients were only obtained for the variables integrated in the factor 2 of the PCA in Coldecarrera population. No further morphometric traits exhibited significant regression coefficient for any of the studied populations (Table 4.1.6).

## Discussion

Several features are involved in floral traits selection, with legitimate pollinators and nectar robbers being two of the key factors in the dynamics of this assortment (Irwin *et al.* 2001; Irwin 2006). The behaviour and preferences of visitors and their efficiency in pollen transfer has major impacts in the reproductive outputs of the plant and subsequently in the selection of several traits.

The elaborated corolla morphology of *P. vayredae* results in a long corolla tube where nectar is enclosed. Consequently, only a group of visitors with long proboscides are able to access the nectar through a legitimate visit, with the remaining visitors piercing the corolla tube or the upper sepal to access the nectar. This is similar to what was observed in other studies on tubular flowers, where illegitimate visits by nectar robbers were a frequent phenomenon (*e.g.*, Navarro 1999; Urcelay *et al.* 2006). As nectar robbing was a steady element in all the studied populations (Chapters 2.1 and 4.2) and as it can affect the pollen exportation and fruit production in this species (Chapter 4.3), nectar robbers might play a major role in further plant-visitors interactions, being potentially involved in selective pressures over *P. vayredae* floral traits.

The existence of floral traits variability is considered the first step for selection by floral visitors (Herrera 1990, 1996). Flowers of *P. vayredae* exhibited considerable variability in several floral characters, with the corolla traits being generally positively correlated with each other. Thus, it is plausible that the increase in a corolla trait will result in a general increase of the corolla size and *vice versa*. This is in accordance with the assumption of better floral integration in species that depend on pollen vectors (Berg 1959; Armbruster *et al.* 1999). Furthermore, significant differences among the studied populations were observed for all the analysed characters, with exception of the nectar gland. This character presented considerable intra-population variability, resulting in a lack of significant differences among populations. Nectar gland dimension was positively correlated with nectar production, thus being a good measure to infer the main floral reward offered by the flowers of *P. vayredae*. This is in accordance with other studies among several species, where a good fit between flower length and volume of secreted nectar was also found (*e.g.*, Galetto and Bernardello 2004). As the nectar gland was not correlated with the remaining characters in any of the studied populations, it seems that this floral trait varied independently within the flower, decoupled from the remaining corolla characters.

In general, successfully pollinated flowers of *P. vayredae* were smaller and produced larger nectar rewards. Moreover, they were less subjected to nectar robbing. Apparently,

bigger flowers were more prone to be elected by nectar robbers, with legitimate pollinators being more likely to avoid these intensively robbed flowers (e.g., Irwin and Brody 1999). On the other hand, smaller flowers were less subjected to nectar robbing and presented higher female fitness. Therefore, in this species it seems that the selection of some floral traits is indirectly driven by the nectar robbers, by potentially changing the pollinator's behaviour. If so, the populations with higher nectar robbing will present smaller flowers. Actually, during the year of 2005, nectar robbing was more intense in Coldecarrera, the population that presents flowers with smaller size. The robber's preferences for larger flowers has already been observed in previous studies involving artificial flowers (Lara and Ornelas 2001), and in *Campsidium valdivianum* (Phil.) Bull (Urcelay *et al.* 2006) and *Duranta repens* L. (L. Navarro and R. Medel, unpublished data). Although variable, it has been previously shown that the indirect interactions of robbers on legitimate pollinators are important in driving the selection for some floral traits (Irwin 2006).

The correlation analyses within each population were not so straightforward, and different floral traits involved in successful pollination emerged. Nevertheless, the nectar gland, and thus the floral rewards, played an important role on female fitness, mainly in Montmajor and Coldecarrera populations, with individuals with bigger nectar glands being generally selected. Also, in accordance with the exposed above and despite of the low correlation coefficient, the behaviour of nectar robbers seems to have influenced the subsequent behaviour of legitimate pollinators. This was especially notorious in Coldecarrera population, where robbers preferred flowers with bigger floral traits, while pollinators, which were negatively affected by nectar robbing, preferred flowers less subjected to robbing, *i.e.*, selection for smaller flowers. In Serrat dels Boixos, a complex correlation with floral traits was observed, with nectar robbing being an outside feature during 2005. On other hand, in Montmajor, robbers did not show any preference for the studied floral traits and, as a result, no significant effect of corolla traits in female fitness was detected. In this population, flowers with larger rewards and less subjected to nectar robbing were the ones presenting higher female fitness. Nevertheless, the selective pressures will vary along time according with the abundance and behaviour of nectar robbers and legitimate pollinators, with further investigations involving absolute frequencies of both types of visitors being needed to confirm the proposed hypothesis of indirect negative selection of floral traits mediated by nectar robbers.

Despite several studies searched for the contribution of many factors on phenotypic selection, nectar robbing has been frequently disregarded (but see Irwin 2006). In the present study, although differences were obtained among populations, the results clearly

revealed that nectar rewards and nectar robbing played an important role in the interactions between *P. vayredae* flowers and its legitimate pollinators. Thus, the contribution of nectar robbing, together with several other factors (e.g., herbivory, Herrera *et al.* 2002; Gómez 2003; Sánchez-Lafuente 2007; abiotic factors, Herrera 1993; Giles *et al.* 2006), cannot be overlooked and must be considered in future studies (Irwin 2006). Furthermore, whereas phenotypic variation has a genetic basis and thus, if populations are able to respond to selection, should also be assessed to further understand the mechanisms of floral evolution.

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## 4.2 Spatio-temporal variation in floral visitor's assemblage in *Polygala vayredae* Costa (Polygalaceae)

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## Abstract

The present study describes the interactions between *Polygala vayredae*, a narrow endemic species from pre-Pyrenees, and its floral visitors. Analysis of spatio-temporal variability of floral visitor's assemblages in three populations during three consecutive years, together with studies on their foraging behaviour and effectiveness in pollination were performed. The flowers of *P. vayredae* were visited by a diverse array of insects, overcoming 24 different species. Nonetheless, only four species were legitimate efficient pollinators, moving downwards the keel and entering in contact with the reproductive structures when probing for nectar or pollen. The remaining species behaved as nectar robbers, secondary nectar robbers or nectar thieves. The efficiency/inefficiency of floral visitors on transferring pollen was closely linked with the floral features, as *P. vayredae* flowers present a long and closed corolla tube that greatly restricts the access to floral rewards and to sexual structures. Among the efficient pollinators, two different groups were observed according with their foraging behaviour: the nectarivorous long-tongued bees *Bombus pascuorum* (queens) and *Anthophora* sp., and the pollen collectors *Eucera longicornis* and *Halictus* sp. Taking into account that these insects are generalists, exploiting other resources along their lifespan, and considering the specialized structure of the flower, plant-pollinator interactions in *P. vayredae* point to an asymmetric specialization. No significant differences were observed in the amount of pollen deposited after one visit by each species, but significant differences were observed on the foraging behaviour of the two groups. Legitimate pollinators deposited larger amounts of pollen per visit than necessary, leading to significant pollen losses. Significant variation in the visitor's assemblage and abundance was observed at both spatial and temporal scales, with efficient pollinators being generally scarce. As a consequence, the reproductive outcome was low and significantly variable among populations and years. The implications of these plant-animal interactions are further discussed.

**Keywords** legitimate visitors; long-tongued bees; floral rewards; nectar robbers; nectar thieves, plant-animal interactions; pollination efficiency; pollinators

## **Introduction**

Plant-pollinator interactions are one of the most important and variable mutualisms in nature. The diversity of floral visitor's assemblages in plant-pollinator interactions has major consequences on the plant reproductive outcome (*e.g.*, Pellmyr and Thompson 1996; Traveset and Sáez 1997; Moeller 2005). Multiple floral visitors may show variation in their foraging strategy (nectar or pollen collectors), visitation rate, pollen removal and deposition, and/or handling time. These features will affect the quantitative and qualitative components of pollination, ultimately reflecting different levels of effectiveness, and thus specific contributions to the plant fitness (through both seed production and seed quality). Also, as floral visitors may vary in abundance and efficiency between years and sites (*e.g.*, Traveset and Sáez 1997; Fenster and Dudash 2001; Billick and Tonkel 2003; Moeller 2006), studies of temporal and spatial variability in visitor's assemblage and its effects on plants reproductive outcome are also essential to understand the ecological dynamics of plant populations and communities, as well as the patterns of pollinator-mediated selection (Schemske and Horvitz 1984; Herrera 1988; Eckhart 1992; Schemske *et al.* 1994; Ashman *et al.* 2004).

Determining the relative importance of spatial vs. temporal variation of pollinator-mediated selection is the basis of more comprehensive studies on the distribution of phenotypes across natural populations. While spatial variation in plant-pollinator interactions has been shown to promote divergent evolution of floral traits (Herrera *et al.* 2002a), temporal variation will limit the potential for divergent evolution (Caruso *et al.* 2003). Despite temporal and spatial variation have been repeatedly documented separately (*e.g.*, Schemske and Horvitz 1989; Ashman and Stanton 1991; Moeller 2005), only recently the analysis of the variation in floral visitors over both time and space has received some attention (see Herrera 1988; Horvitz and Schemske 1990; Guitián *et al.* 1996; Gómez and Zamora 1999; Fenster and Dudash 2001; Moeller 2005; Price *et al.* 2005; and references therein). Furthermore, other biotic components, such as competition, herbivory or nectar robbing, as well as abiotic factors of the environment, should also be considered when analysing the consequences of plant-pollinator interactions on plant ecology and evolution (Herrera 1993; Caruso 2000; Herrera *et al.* 2002b; Gómez 2003; Strauss and Irwin 2004; Irwin 2006). The studies on the pollination of flowering plants by animals are also fundamental for the conservation of pollination interactions, especially in systems under increasing risks of anthropogenic activities, such as endangered or narrow endemic species suffering habitat fragmentation, changes in

land use, and/or invasion of non-native plants and animals, among others (Kearns *et al.* 1998).

The present study evaluates both quantitative and qualitative components of a plant-visitor system, and its variation at a spatial (among three populations) and temporal (over three years) scale. The study focuses on *Polygala vayredae* Costa (Polygalaceae), a narrow endemic species with a self-incompatibility system that strictly depends on pollination vectors to set fruits and that suffers pollen limitation due to pollinator's scarcity (Chapter 2.1). Facing this information, it is fundamental to gather data on its floral visitors and determine its effective pollinators in an effort to understand the reproductive ecology and develop adequate conservation measures in this species. Therefore, the main objectives of this study were: (1) to determine the floral visitor's assemblage of *P. vayredae*; (2) to assess its foraging behaviour (by assessing the type of visit, number of visited flowers, handling time and distances of flight within and between clusters) and efficiency (by evaluating specific pollen depositions over the stigmas and presence of pollen over the insect body); (3) to evaluate the spatio-temporal variation in visitation pattern by different floral visitor's; and finally, (4) to understand how the efficient pollinators affect the reproductive success of this protected species through the analysis of both male and female fitness.

## **Materials and methods**

### *Plant and study area*

*Polygala vayredae* is a small shrub with an early flowering period (April to May). It forms dense clusters resulting from shoots produced annually from a rootstock, which greatly difficult the individualization of distinct plants. This species is endemic from Oriental pre-Pyrenees occurring in few dense populations distributed in approximately 12 km<sup>2</sup> in Alta Garrotxa (Girona, Catalunya, Spain) between 550 and 1,070 m of altitude. Ramets produce 1-3 papilionate flowers, with bright pink perianth and yellow sulphur keel. Flowers are large ( $16.2 \pm 0.93$  mm long) and live for  $8 \pm 1.1$  days, offering nectar as a reward at the base of the corolla tube ( $14.6 \pm 0.76$  mm long), mainly during the first three days of the flower anthesis (Chapter 2.1). Each flower produces  $5,428 \pm 1,046.3$  pollen grains, possess two ovules and can only develop seeds after outcrossing pollination, due to an incompatibility system at the stigmatic level. Nonetheless, despite of the attractive flower features, it has been previously observed that pollen limitation plays an important role on the final reproductive outcome of the plant (Chapter 2.1).

The study was carried out in Alta Garrotxa during three consecutive springs (2005-2007) on the three main populations of *P. vayredae*: (1) Montmajor, (2) Serrat dels Boixos and (3) Coldecarrera (for further details see Chapter 2.1 and Fig. 1.1).

#### *Floral visitor's assemblage and foraging behaviour*

To determine the floral visitor's assemblage and behaviour, direct observations of flower visitors were made in each population. The observations were performed during the flowering peak in several randomly selected areas of approximately 2 m<sup>2</sup>, where all the flowers could be easily monitored. The observer was positioned at approximately 1 m from the study area, being able to monitor all the floral visitors without disturbing their foraging behaviour. Visits were recorded during series of 15 min of surveillance sessions at different hours of the day (from 0800 to 1800 h, GMT). A total of 1,155 censuses evenly distributed per population and year were performed, corresponding to 289 h of net observation. During each session the following variables were registered: visiting species and type of visit, total number of flowers visited per cluster, handling time and foraging distances within and between clusters. All the floral visitors interacting with *P. vayredae* were recorded and further characterized following Inouye (1980) as: legitimate visitors (L), nectar robbers (R), secondary nectar robbers (SR) and nectar thieves (T). Additionally, legitimate visitors that were not able to move the keel downwards, and thus, did not enter in contact with the reproductive organs (for illustrations see Chapter 3.1), were further classified as inefficient legitimate visitors (L<sub>i</sub>). The flower visitation rate of each insect species (number of flowers visited per 15 min; Herrera 1989) was assessed as the total number of visited flowers per cluster divided by the total number of surveillance sessions. The probability of a flower to receive a visit from an efficient or an inefficient visitor was also calculated. At the end of field observations, specimens of each floral visitor were collected for identification. Measurements of proboscis and body length were performed under a stereo microscope using an ocular micrometer. Voucher specimens were deposited at the Department of Biology, University of Aveiro.

#### *Floral visitor's foraging efficiency*

The main components of visitor effectiveness, *i.e.*, the frequency of interactions, per-visit pollen deposition and presence of pollen over insect body (Herrera 1987, 1989; Fishbein and Venable 1996; Gómez and Zamora 1999), were analysed for each visitor species of *P. vayredae*. In each studied population along the three years, the frequency of interactions was calculated following Herrera (1989) for 15 min by multiplying insect



abundance (number of insects per cluster) and flower visitation rate. The presence and placement of pollen over the insect body was evaluated under a stereo microscope in all the collected floral visitors. The amount of pollen deposited over the stigma by each floral visitor was evaluated on the surveillance areas by emasculating and bagging up to 100 randomly selected flowers prior to anthesis. After flowering and during surveillance periods, flowers were unbagged for controlled open pollination. After receiving a visit, flowers were immediately bagged and identified with the floral visitor. Each visited flower was collected 24 h after, and harvested in ethanol 70% for counting pollen grains over the stigmatic papillae. All the unvisited flowers were bagged when censuses finished. This procedure was repeated until a representative set of flowers visited by each floral visitor was obtained. At the laboratory, pistils of visited flowers were softened with sodium hydroxide 8 N for 4 h, stained with aniline blue 0.05% overnight and squashed in a drop of glycerine 50% (Dafni *et al.* 2005). Finally, pistils were observed through an epifluorescence microscope with a UV-2A filter cube (330-380 nm excitation) and the total number of pollen grains over the stigma was counted.

#### *Measurements of plant male fitness in each population*

Male efficiency was evaluated through the following parameters: (1) the proportion of pollen grains that reach one stigma of another flower (number of pollen grains in each stigma divided by the mean number of pollen grains produced by the flower); (2) the proportion of pollen grains that reach one stigma of another flower and emit pollen tubes through the style (number of pollen grains with pollen tubes in the style divided by the mean number of pollen grains produced by the flower); and (3) the proportion of pollen grains able to germinate and pass the stigmatic papillae with respect to the total amount of pollen deposited by the pollinator. Whereas the first two proportions provide a measure of pollen grains success to reach one flower, the third provides a measure of the success and competition among the pollen grains deposited by the pollinator. For this, 80 to 100 flowers collected during spring of 2005 from all populations were harvested in ethanol 70%. At the laboratory, pistils were treated as described above and observed through an epifluorescence microscope with a UV-2A filter cube. The total number of pollen grains over the stigma and the number of pollen tubes able to germinate and grow throughout the stigmatic papillae and style were recorded.

*Measurements of plant female fitness in each population*

Reproductive success, given by the fruit and seed production, was evaluated in all the populations along the three years of study. For this, in each population, up to 220 flowers were randomly selected and marked. When mature, the total number of fruits and seeds produced was recorded.

*Nectar standing crop*

Nectar standing crop was assessed during spring of 2006 in Coldecarrera population. For this, at each hour of the day (from 0900 to 1800 h, GMT), up to 30 flowers randomly selected along the population were used to measure nectar availability (volume and sugar concentration). The volume of available nectar was measured with a capillary micropipette and sugar concentration was determined (in w/w %) with a portable refractometer.

*Statistical analysis*

The variation on the abundance of floral visitors among populations, years and efficient vs. inefficient visitors was analysed using Generalized Linear/Nonlinear Models (GLZ), with the dependent variable, visitor abundance, adjusted to a Poisson distribution and a logarithmic link function for model responses. The Type 3 Likelihood Ratio test of effects was computed.

Regarding visitor efficiency variables, the number of deposited pollen grains on the stigma by each efficient legitimate pollinator was analysed with a one-way ANOVA, while the number of visited flowers per cluster, handling time, and flight distances were analysed with a Kruskal-Wallis one-way ANOVA on ranks, followed by pairwise multiple comparison using the Dunn's method. A similar procedure was followed to assess differences among populations in the proportion of pollen grains reaching one stigma and in the proportion of pollen grains emitting pollen tubes.

Differences in fruit set and seed ovule ratio among populations and years were analyzed using GLZ, with the dependent variables (categorical data) approximated to a binomial distribution and a logit link function for model responses. The Type 3 Likelihood Ratio test of effects was computed. The probability of *P. vayredae* flowers to receive the visit of an efficient and inefficient floral visitor was determined for each surveillance period (15 min), through the ratio between the total number of flowers visited by each visitor group and the total number of flowers under observation (presented as mean and standard deviation of the mean for all the studied populations and years). Correlations

among fruit set or seed ovule ratio and the probability to receive a visit from an efficient and inefficient visitor were assessed through a Pearson correlation.

Correlations among the nectar volume, nectar concentration and cumulative number of visited flowers (assuming that nectar is re-synthesised during the night) along the several hours of the day were assessed through a Spearman rank order correlation.

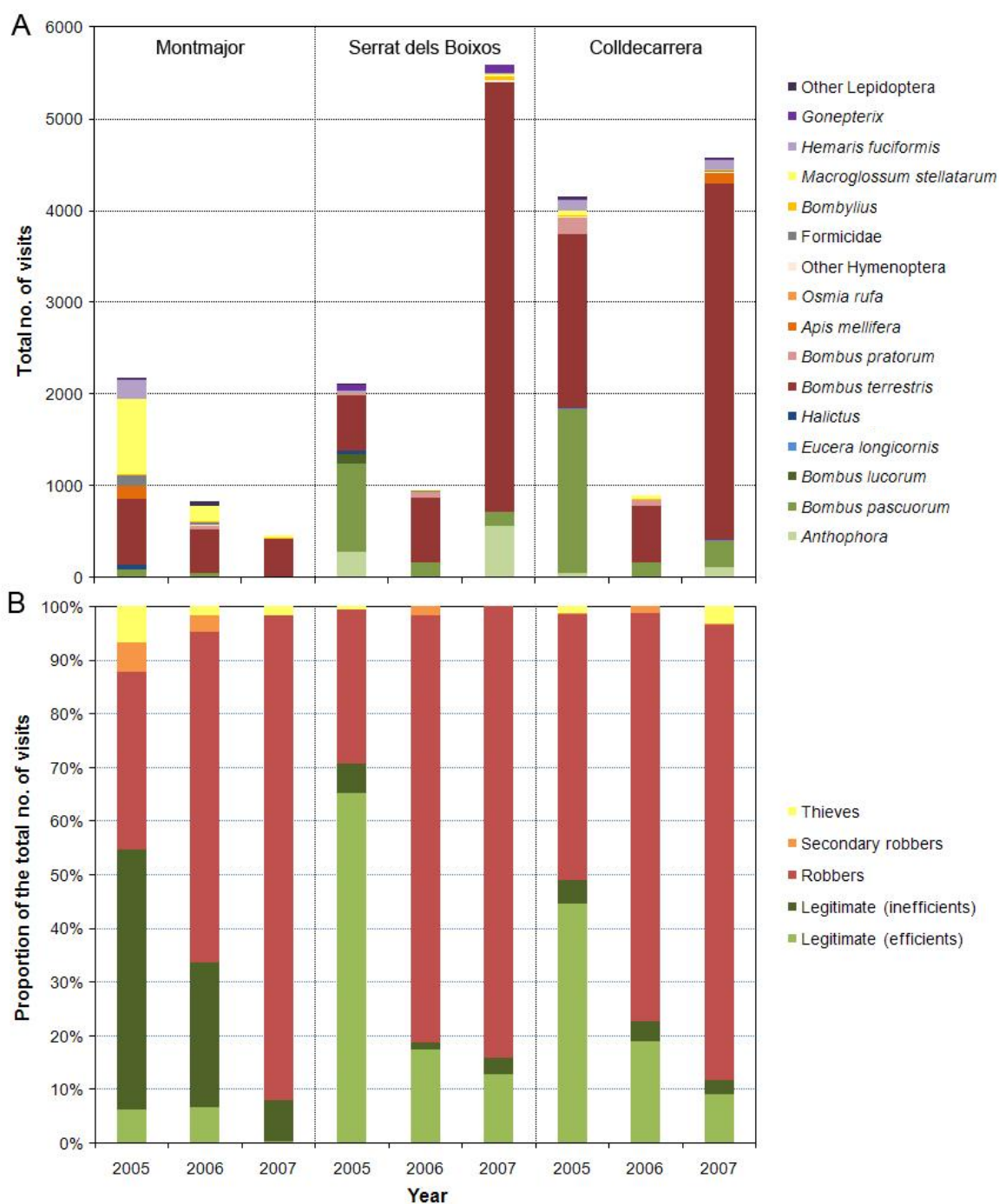
## Results

### *Floral visitor assemblage and foraging behaviour*

During the three years of study and along three populations a total number of 21,824 floral visitor interactions performed by 967 individuals were observed, with the flowers of *P. vayredae* being visited by a diverse and variable array of visitors. At least 27 insect species belonging to orders Hymenoptera (59.3% of the total species), Lepidoptera (37.0%) and Diptera (3.7%) were observed (see Appendix 4.2.1). The most abundant species was *Bombus terrestris*, which represented 64.2% of the overall floral visits, followed by *B. pascuorum* queens (16.8%), *Macroglossum stellatarum* (5.2%) and *Anthophora* sp. (4.7%) (Figs. 4.2.1 and 4.2.2A). The remaining 23 species accounted with only 9.1% of the total visits (Fig. 4.2.2A and Appendix 4.2.1). Nonetheless, the floral visitors presented different foraging behaviours when facing the long and close corolla tube of *P. vayredae* flowers, with the majority searching for nectar (the exception being the pollinivorous species, *Eucera longicornis* and *Halictus* sp.). Thus, from the 27 species, only 18.5% were efficient legitimate pollinators, with the remaining 81.5% acting as nectar robbers (22.3%; e.g., *B. terrestris* and *B. pratorum*), secondary nectar robbers (11.1%; e.g., Formicidae), nectar thieves (18.5%; e.g., *Apis mellifera*) or inefficient legitimate visitors unable to move the keel and contact with reproductive organs (29.6%; e.g., the majority of Lepidoptera and *Bombylius* sp.) (Figs. 4.2.1 and 4.2.2B).



**Fig. 4.2.1** Main floral visitors of *Polygala vayredae* flowers: (A) *Bombus terrestris* queen, (B) *B. pascuorum* queen, (C) *Macroglossum stellatarum*, and (D) *Anthophora* sp.



**Fig. 4.2.2** Floral visitor's assemblage of *Polygala vayredae*: spatial and temporal variation in (A) floral visitor's assemblage, and (B) floral visitor's type.

The spectrum of floral visitors of *P. vayredae* varied considerably both in time and space. Significant differences in insect abundance were observed among factors (year, population and visitor type) and in their interactions (Table 4.2.1). The significant differences observed in the interactions year  $\times$  population, population  $\times$  visitor type, and year  $\times$  population  $\times$  visitor type resulted from variable visitor's abundance. The lowest

values of insect abundance were observed during 2006 in any population, and in Montmajor population in general (Fig. 4.2.2A). Furthermore, the abundance of efficient and inefficient visitors also varied significantly, with inefficient visitors (*i.e.*, nectar robbers, secondary nectar robbers, nectar thieves and legitimate inefficient visitors) being much more abundant (except in Serrat dels Boixos during 2005). This can be clearly observed in Figure 4.2.2A, where it is also notorious that *B. terrestris* (nectar robber) was the main visitor of *P. vayredae* during 2006 and 2007. Among the legitimate pollinators of this species, and not considering the Montmajor population that clearly presents pollinators limitation, *B. pascuorum* was present in Serrat dels Boixos and Coldecarrera along the three years, despite of its highly variable abundance (Fig. 4.2.2A). The analysis of the abundance of each type of visitor revealed a decrease in efficient visitors along the three years in Serrat dels Boixos and Coldecarrera populations (Fig. 4.2.2). In Montmajor, efficient visitors were scarce in any of the monitored years, being always below 10% of the total number of visits (Fig. 4.2.2B).

**Table 4.2.1** Results of the Generalized Linear/Nonlinear Model performed on insect abundance for the efficient and inefficient visitors for three years and three populations.

Effect	df	$\chi^2$	P
Year	2	116.07	0.0000
Population	2	87.38	0.0000
Visitor	1	275.93	0.0000
Year × Population	4	155.89	0.0000
Year × Visitor	2	5.33	0.0697
Population × Visitor	2	14.01	0.0009
Year × Population × Visitor	4	85.31	0.0000

As expected, data on floral visitation rate is in accordance with the floral visitor's abundance, *i.e.*, within nectar larcenists, the highest visitation rates were observed for *B. terrestris* (12.14) and *M. stellatarum* (0.99), while for pollinators they were observed for *B. pascuorum* (3.17) and *Anthophora* sp. (0.88), with the remaining pollinator species presenting very low visitation rates (<0.1; Table 4.2.2).

**Table 4.2.2** Behavioural features of the floral visitors of *Polygala vayredae*.

Visitor species	Type of visit	Proboscis size (mm)	Length of insect body (mm)	Visitation rate	No. flowers visited per cluster	Handling time (sec)	Deposited pollen	Distances within a cluster (cm)	Distances between clusters (cm)
Order Hymenoptera									
<i>Anthophora</i> sp.	L	10.4 ± 2.09	15.6 ± 1.18	<b>0.884</b>	12 ± 11.5	2.6 ± 2.25	86 ± 65.3	19.2 ± 19.51	246 ± 97.8
<i>Bombus terrestris</i>	R	5.8 ± 0.34	17.3 ± 1.64	<b>12.140</b>	36 ± 34.0	3.3 ± 3.20	0	17.6 ± 20.47	550 ± 169.6
<i>Bombus pascuorum</i>	L	8.9 ± 0.81	16.8 ± 0.91	<b>3.172</b>	22 ± 20.7	3.0 ± 2.19	122 ± 80.3	14.2 ± 15.88	595 ± 230.3
<i>Bombus lucorum</i>	L	5.9	17.1	0.093	36 ± 22.9	3.1 ± 4.27	111 ± 18.4	16.6 ± 13.87	>>
<i>Bombus pratorum</i>	R	5.7 ± 0.78	10.6 ± 1.91	0.280	16 ± 16.0	3.2 ± 2.54	0	27.3 ± 21.63	>>
<i>Bombus lapidarius</i>	R	3.2 ± 0.64	16.9 ± 0.04	0.005		3.9 ± 4.79	0		
<i>Bombus hortorum</i>	R			0.016			0		
<i>Psithyrus</i> sp.	R	1.9	12.5	0.003			0		
<i>Apis mellifera</i>	T	3.1 ± 0.35	12.3 ± 0.35	0.238	12 ± 15.1	4.3 ± 3.27	0	23.2 ± 18.43	217 ± 54.7
<i>Eucera longicornis</i>	L	4.6	11.8	0.025	7 ± 4.9	8.8 ± 6.22	97 ± 45.8	28.9 ± 20.72	>>
<i>Halictus</i> sp.	L	0.9	7.6	0.081	5 ± 3.3	7.3 ± 7.42		27.4 ± 20.31	>>
<i>Osmia rufa</i>	T	4.7	13.8	0.054	8 ± 3.5	3.4 ± 2.25	0	19.4 ± 19.85	>>
<i>Xylocopa violacea</i>	R			0.005		3.2	0		
Formicidae	SR	-	<0.7	0.158	3 ± 3.8		0	-	-
Order Diptera									
<i>Bombylus</i> sp.	L <sub>i</sub>	6.2 ± 0.71	8.8 ± 1.42	0.079	3 ± 1.8	1.9 ± 1.47	0	47.9 ± 18.74	>>
Order Lepidoptera									
<i>Macroglossum stellatarum</i>	L <sub>i</sub>	26.0 ± 1.44	29.6 ± 1.64	<b>0.989</b>	13 ± 12.4	1.9 ± 2.68	0	24.4 ± 23.18	254 ± 107.6
<i>Hemaris fuciformis</i>	L <sub>i</sub>	14.6 ± 1.32	29.5 ± 0.96	0.416	25 ± 39.5	2.2 ± 2.18	0	24.0 ± 22.03	249 ± 89.7
<i>Gonepteryx rhamni</i>	L <sub>i</sub>	19.6 ± 1.04	22.4 ± 1.31	0.142	5 ± 5.7	13.9 ± 11.76	0	61.1 ± 32.84	>700
<i>Gonepteryx cleopatra</i>	L <sub>i</sub>			0.029	3 ± 1.8	17.0 ± 10.91	0		
<i>Leptidea sinapis</i>	T	<10.0	14.8 ± 0.35	0.004	2 ± 1.2		0		>>
<i>Papilio machaon</i>	T	13.5	24.5	0.017	10 ± 5.7	5.7 ± 5.07	0		>>
<i>Pyronia</i> sp.	T	<10.0	16.5	0.022	3 ± 2.0	10.1	0		
<i>Aglais urticae</i>	L <sub>i</sub>			0.001			0		
<i>Vanessa atalanta</i>	L <sub>i</sub>			0.008	2 ± 1.3		0	40.3 ± 21.71	>>
<i>Vanessa cardui</i>	L <sub>i</sub>			0.033	5 ± 3.1	8.9 ± 6.87	0		

Notes: Type of visit: L – legitimate visitor, L<sub>i</sub> – legitimate inefficient visitor, R – nectar robber, SR – secondary nectar robber and T – nectar thief. The visitation rate of the four main floral visitors is highlighted in bold. Deposited pollen is given by the number of pollen grains deposited by a specific visitor over the stigmatic papillae after one visit. >> – insect was lost of sight.

*Floral visitor's foraging efficiency*

Five floral visitors were observed pollinating the flowers of *P. vayredae*: *Anthophora* sp., *B. pascuorum* queens, *B. lucorum*, *E. longicornis* and *Halictus* sp. The remaining visitors explored the flowers without entering in contact with the reproductive structures and thus, were not involved in pollination (Table 4.2.2). Thus, as expected, pollen of *P. vayredae* was only found on the body of efficient legitimate pollinators; the remaining visitors did not carry pollen in their bodies nor deposited pollen in the stigmas. Among the four species of pollinators for which pollen deposition was assessed, no significant differences in the amount of pollen deposited over the stigmatic papillae were observed ( $F = 0.840$ ,  $P = 0.478$ ). On average the pollinators deposited  $110 \pm 72.6$  pollen grains after one visit (Table 4.2.2).

*Anthophora* sp. and *B. pascuorum*, the two main pollinators of *P. vayredae* in the studied populations, presented the longest proboscides (excluding Lepidoptera) and robust bodies, enabling them to trigger the flower and reach the nectar without robbing. *E. longicornis* and *Halictus* sp., when foraging for pollen, exposed the stigmatic region, being directly involved in pollination due to the close proximity between the site of pollen presentation and the stigmatic papillae (for illustrations see Chapter 3.1). Despite of its relatively short proboscis, considering the length of *P. vayredae* corolla tube, *B. lucorum* behaves as a legitimate visitor. Unfortunately, due to its scarcity it was difficult to thoroughly evaluate if this species always behaves as a pollinator or if changes its behaviour to nectar robber. The remaining Hymenoptera (excluding Formicidae), due to their short proboscides, are not able to achieve nectar in a legitimate way, behaving as primary nectar robbers (by piercing at the base of the corolla where nectar is accumulated), secondary robbers or nectar thieves. By other way the species from Formicidae profit from the existing holes (made by primary nectar robbers) to assess food reward. Lepidoptera, despite visiting the flowers in a legitimate way, present long but thin proboscis and very light bodies, which enable them to reach the nectar without triggering the flower, and thus being excluded from pollination. Like Lepidoptera, *Bombylius* sp. also tries to reach nectar in a legitimate way but its flying behaviour makes it ineligible for the pollination of *P. vayredae*.

Regarding the efficient legitimate pollinators of *P. vayredae* flowers, significant differences in the behaviour of the insects with different foraging strategies (nectarivorous vs. pollinivorous) were observed. The nectar-collecting *B. pascuorum*, *Anthophora* sp. and *B. lucorum* visited a significantly larger number of flowers per patch ( $H = 25.1$ ,  $P < 0.001$ ), spent less time per flower ( $H = 61.8$ ,  $P < 0.001$ ) and moved for shorter distances within

the clusters ( $H = 17.9$ ,  $P = 0.001$ ), when compared with the pollen-collecting *E. longicornis* and *Halictus* sp. (Table 4.2.2). Between clusters, *Anthophora* sp. moved to shorter distances than *B. pascuorum* ( $T = 488.0$ ,  $P < 0.001$ ), while *B. lucorum*, *E. longicornis* and *Halictus* sp. were always lost from the observer sight, potentially moving to farer distances.

**Table 4.2.3** Spatial and temporal variation in the frequency of interaction between *Polygala vayredae* and its floral visitors.

Visitor species	Type of visit	Montmajor			Serrat dels Boixos			Coldecarrera		
		2005	2006	2007	2005	2006	2007	2005	2006	2007
<i>Anthophora</i> sp.	L		<0.001		0.492		<b>1.878</b>	0.007	0.001	0.196
<i>Bombus terrestris</i>	R	<b>1.072</b>	<b>0.840</b>	<b>0.543</b>	<b>2.083</b>	<b>2.001</b>	<b>34.690</b>	<b>2.157</b>	<b>0.860</b>	<b>42.253</b>
<i>Bombus pascuorum</i>	L	0.022	0.025		<b>3.959</b>	<b>0.222</b>	0.178	<b>2.423</b>	<b>0.088</b>	<b>0.744</b>
<i>Bombus lucorum</i>	L				0.030					
<i>Bombus pratorum</i>	R		0.013		0.002	0.028		0.011	0.011	
<i>Apis mellifera</i>	T	0.126		0.001	<0.001					0.060
<i>Eucera longicornis</i>	L							<0.001		0.003
<i>Halictus</i> sp.	L	0.040		<0.001	0.026					
<i>Osmia rufa</i>	T		0.002		<0.001			0.002		0.002
Other Hymenoptera	R		<0.001				0.003	<0.001		0.002
Formicidae	SR	0.138	0.042			0.019		<0.001	0.009	0.006
<i>Bombylius</i> sp.	L <sub>i</sub>	0.001	0.002	0.002			0.016	<0.001	<0.001	0.012
<i>Macroglossum stellatarum</i>	L <sub>i</sub>	<b>2.723</b>	<b>0.217</b>	<b>0.011</b>		0.002	0.006	0.001	0.002	
<i>Hemaris fuciformis</i>	L <sub>i</sub>	0.100			0.004		0.002	0.010		0.013
<i>Gonepteryx</i> sp.	L <sub>i</sub>	<0.001			0.081		0.159	0.002		0.011
Other Lepidoptera	L <sub>i</sub> /T	<0.001	0.031		0.002			0.002		0.010

Notes: The frequency of interaction of the two main floral visitors in each population along the three years of study is highlighted in bold.

Only four species, *B. terrestris*, *B. pascuorum*, *M. stellatarum* and *Anthophora* sp., presented frequencies of interaction higher than one (Table 4.2.3). However, while *B. terrestris* was a regular nectar robber of *P. vayredae* flowers, usually presenting the highest frequencies of interaction, the remainder species only presented values higher than one in specific occasions [in some population(s) of particular year(s)]. Considering the type of visitor, legitimate pollinators presented a highly variable frequency of interaction among populations and years, as a result of their unpredictable abundance and visitation rates. The frequency of interaction of legitimate pollinators was always inferior to that of larcenists, with exception of the year 2005 in Serrat dels Boixos and Coldecarrera, where proportionally, the abundance of *B. pascuorum* and *B. terrestris* was similar (Table 4.2.3 and Fig. 4.2.2A). It should be also noticed that while in those populations one of the two floral visitors with the greatest frequency of interaction was a



legitimate pollinator, in Montmajor two larcenists were the main insects interacting with *P. vayredae* flowers. Furthermore, in the latter population very low frequencies of interactions were generally obtained, as a result of low abundance of floral visitors, particularly efficient pollinators (Table 4.2.3 and Fig. 4.2.2A).

#### *Measurements of plant male fitness in each population*

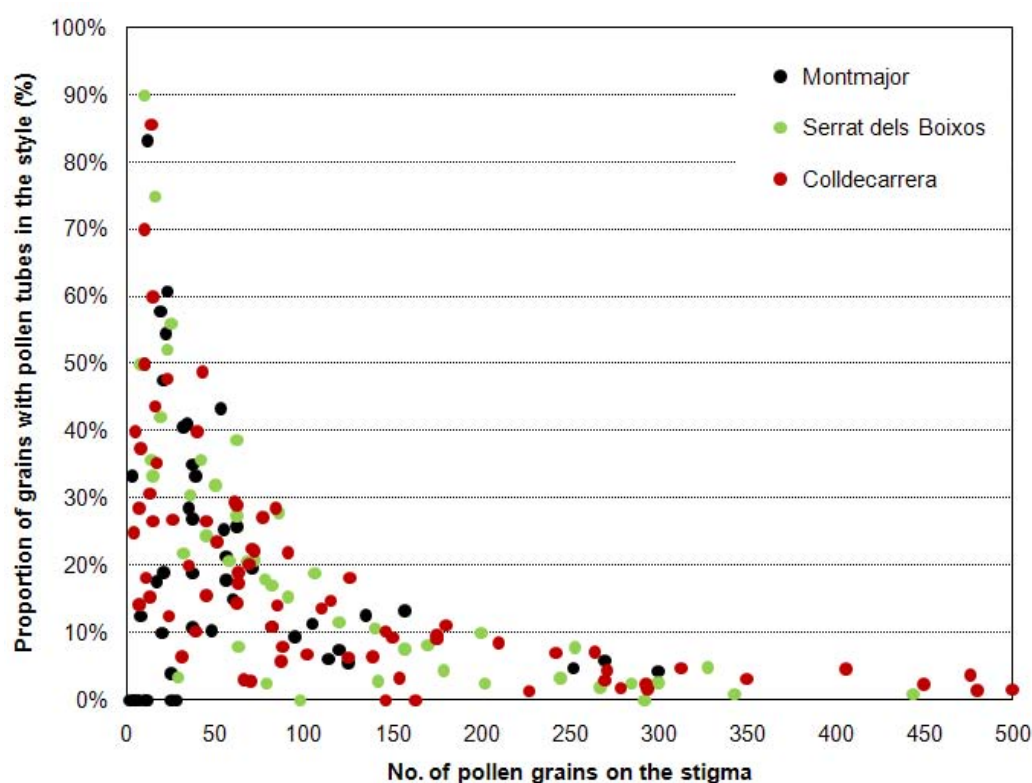
Male efficiency given by the success of pollen grains to reach the stigma and germinate through the style of another flower was evaluated in each population and is presented in Table 4.2.4. Significant differences in the proportion of pollen grains reaching the stigma were obtained among populations ( $H = 14.54$ ,  $P < 0.001$ ), with flowers from Montmajor receiving lower proportions of pollen grains. Nonetheless, when comparing the proportions of pollen grains that emit pollen tubes in the style, no significant differences among populations were observed ( $H = 3.81$ ,  $P = 0.149$ ).

**Table 4.2.4** Male efficiency given by the success of pollen grains to reach the stigma and germinate through the style of another flower for each of the studied populations.

Population	Proportion of pollen grains reaching one stigma (%)	Proportion of pollen grains emitting pollen tubes (%)
Montmajor	$1.1 \pm 1.25^a$	$0.1 \pm 0.11$
Serrat dels Boixos	$2.2 \pm 2.02^b$	$0.2 \pm 0.12$
Colldecarrera	$2.3 \pm 2.35^b$	$0.2 \pm 0.12$
Comparison test	$H = 14.54^{***}$	$H = 3.81$ n.s.

Notes: The proportions were calculated from the mean number of pollen grains produced by the flowers of *P. vayredae* obtained in Chapter 2.1.

The proportion of pollen grains able to germinate and pass the stigmatic papillae with respect to the total amount of pollen deposited by the pollinator is presented in Figure 4.2.3. A similar pattern was observed in all populations, *i.e.*, the male fitness drastically decreased with the increase of pollen load. Consequently, a larger number of pollen grains provided by the insect do not imply that a higher percentage of pollen tubes are developed. For instance, from the mean number of pollen grains deposited by the pollinators after one visit (*i.e.*,  $110 \pm 72.6$  pollen grains) only up to 25% were able to develop pollen tubes in the style (Fig. 4.2.3).



**Fig. 4.2.3** Fitness of the male gametes that achieved the stigmatic papillae of a flower (given by the proportion of pollen grains able to germinate and pass the stigmatic papillae with respect to the total amount of pollen deposited by the pollinator) in each of the studied populations.

#### *Measurements of plant female fitness in each population*

The female fitness measured as fruit set and seed ovule ratio varied significantly among years and populations (Table 4.2.5 and 4.2.6). Furthermore, a significant interaction between years and populations was observed for the seed ovule ratio. Usually, the highest values of fruit and seed production were observed in Coldecarrera, while the lowest ones were obtained in Montmajor. Within each population, female fitness was higher during 2005, with the lowest values being usually obtained in 2007.

Overall, the probabilities of *P. vayredae* flowers to receive a visit from an efficient and/or inefficient insect in a 15 min period were highly variable (Table 4.2.5). In general, the flowers of *P. vayredae* had a higher probability to receive a visit from an inefficient visitor than from an efficient one, in any of the populations and years. Positive correlations between receiving an efficient visitor and both female fitness parameters were obtained ( $R^2 = 0.709$ ,  $P = 0.033$  for fruit set; and  $R^2 = 0.654$ ,  $P = 0.050$  for seed ovule ratio; Table 4.2.5). Nonetheless, unexpectedly, in Serrat dels Boixos the low probability of receiving an efficient visit obtained in 2006 resulted in the second higher value of fruit set and seed

ovule ratio. No clear correlations between the probability of receiving an inefficient visitor and female fitness were observed ( $R^2 = 0.142$ ,  $P = 0.716$  for fruit set; and  $R^2 = 0.116$ ,  $P = 0.767$  for seed ovule ratio; Table 4.2.5).

**Table 4.2.5** Probability of *Polygala vayredae* flowers to set fruits and seeds, and to receive efficient and inefficient floral visits in a 15 min period along the three studied populations during 2005, 2006 and 2007.

Population	Year	Fruit set	Seed ovule ratio	Probability of efficient visit (%)	Probability of inefficient visit (%)
Montmajor	2005*	7.4	4.8	$0.6 \pm 2.09$	$6.5 \pm 11.80$
	2006	3.0	1.8	$0.2 \pm 0.78$	$2.0 \pm 5.09$
	2007	1.9	0.9	$0.01 \pm 0.05$	$2.4 \pm 9.18$
Serrat dels Boixos	2005*	28.9	19.5	$5.6 \pm 8.69$	$3.1 \pm 7.08$
	2006	22.3	16.7	$0.7 \pm 2.21$	$2.6 \pm 4.64$
	2007	14.8	12.3	$1.5 \pm 2.86$	$10.6 \pm 14.93$
Coldecarrera	2005*	47.6	36.9	$3.1 \pm 8.20$	$4.1 \pm 11.60$
	2006	21.0	15.3	$1.1 \pm 4.08$	$4.1 \pm 12.44$
	2007	26.0	17.5	$2.7 \pm 7.51$	$17.9 \pm 23.83$

Notes: The probability to receive an efficient or inefficient visit is given as mean and standard deviation of the mean.

\*Data of fruit set and seed ovule ratio from 2005 was obtained from Chapter 2.1.

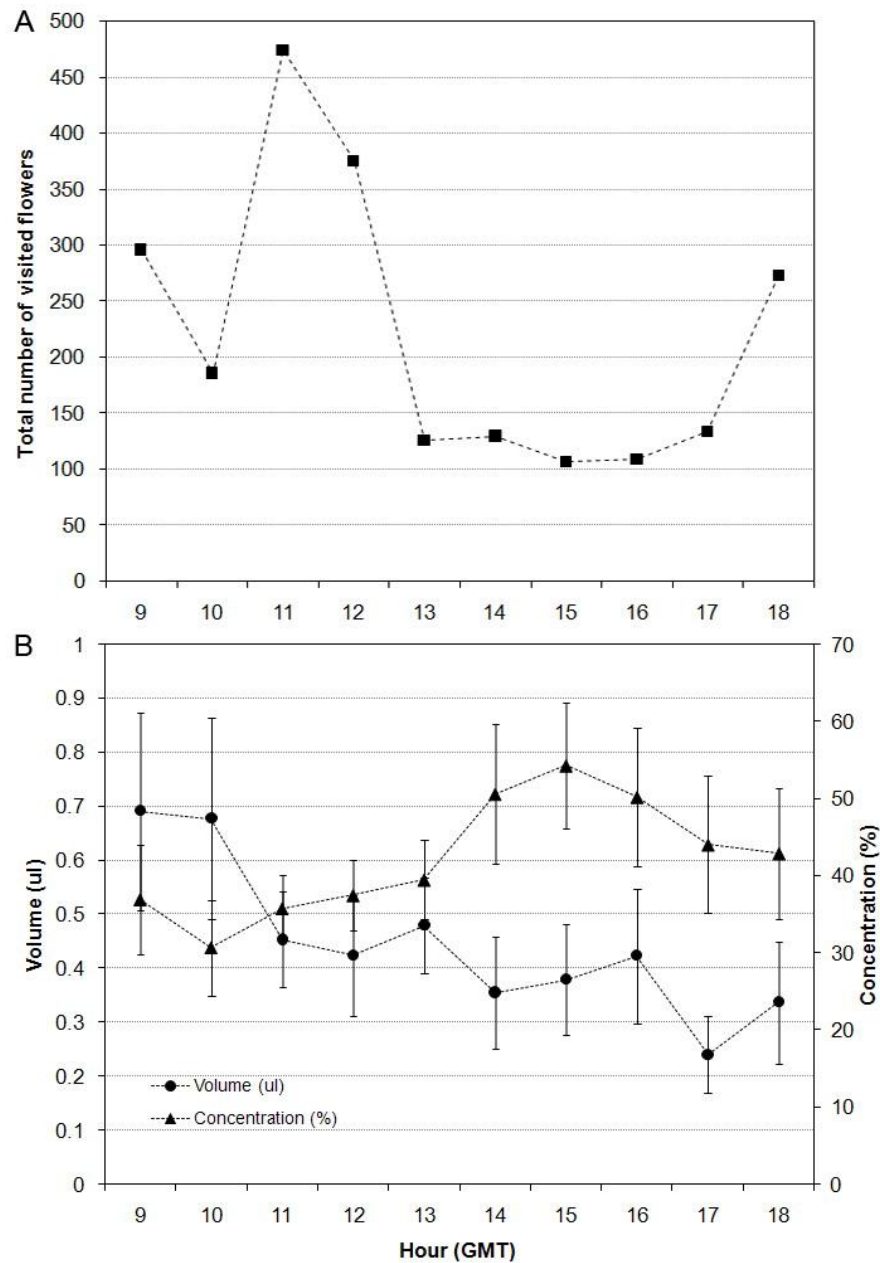
**Table 4.2.6** Results of the Generalized Linear/Nonlinear Model performed on fruit set and seed ovule ratio for three years and three populations.

Effect	df	Fruit set		Seed ovule ratio	
		$\chi^2$	$P$	$\chi^2$	$P$
Year	2	36.20	0.0000	47.59	0.0000
Population	2	130.32	0.0000	195.30	0.0000
Year $\times$ Population	4	5.92	0.2055	12.17	0.0161

### Nectar standing crop

The volume of nectar was found to be negatively correlated with the hour of the day ( $R^2 = -0.857$ ,  $P = 0.002$ ), while the sugar concentration evolved in the opposite direction ( $R^2 = 0.681$ ,  $P = 0.030$ ) (Fig. 4.2.4B). The higher visitor's activity, measured as the total number of flowers visited per hour, was registered between 1100 and 1300 h (GMT) (Fig. 4.2.4A).

The correlation between the cumulative number of visited flowers and nectar volume showed that the volume of available nectar decreased through the day with the increase in the number of visited flowers ( $R^2 = -0.911$ ,  $P < 0.001$ ).



**Fig. 4.2.4** Number of visited flowers and nectar standing crop of *Polygala vayredae* registered along the day: (A) total number of visited flowers, and (B) volume and concentration of the available nectar (values are given as mean and standard deviation of the mean). Observations were performed during 2006 in Coldecarrera population.

## Discussion

*Polygala vayredae* possess a diverse array of floral visitors mainly composed by Hymenoptera and Lepidoptera. However, the attractive flowers of this species not only caught the attention of pollinators, but also of a large number of larcenists. Indeed, in the surveyed area, legitimate pollinators were restricted to four species (not considering *B. lucorum*, see results), from which two (the long-tongued *B. pascuorum* queens and *Anthophora* sp.) were predominantly more abundant than the others (the pollinivorous *Halictus* sp. and *E. longicornis*). In contrast, larcenists were present in a larger number and thus, represented a constant feature in this plant. In each population, the floral visitor's assemblage of *P. vayredae* was composed of a few highly abundant species, usually a pollinator and a larcenist, with the remaining visitors being comparatively rare. Due to the large incidence of larcenists, nectar robbing was frequently observed in all populations. Similar observations have been performed in several other nectar rewarding species, with nectar robbing potentially playing an important role in further plant-animal interactions, having positive, neutral or negative effects on the plant reproductive outcome (e.g., Arizmendi *et al.* 1996; Irwin and Brody 1999; Maloof and Inouye 2000; Navarro 2000; Zhang *et al.* 2007). In *P. vayredae* it has been suggested that nectar robbing has important indirect and negative effects on plant fecundity, through both male and female functions, as a result of changes in the behaviour of legitimate pollinators, which visit less flowers and export pollen to a lower distance in robbed flowers than in unrobbed ones (Chapter 4.3).

The pollinator species observed in *P. vayredae* flowers presented two different behaviours according with their foraging strategy. While nectarivorous species confidently exploited a larger number of flowers, moving between neighbouring ramets and spending less time per flower, pollinivorous species visited fewer flowers, moving to ramets located at greater distance, but spending more time per flower as they entered several times during pollen collection. This contrasting foraging behaviour did not seem to influence the amount of pollen deposited over the stigmas after one visit, resulting in apparently similar efficiency rates of pollination among species. However, due to the close proximity of male and female structure in this plant (for details see Chapter 3.1), the foraging attitude of pollinivorous when exploiting a flower may affect the extent of self pollen deposition and thus, the quality of the received pollen. On the other hand, when flying for larger distances these insects are able to export the pollen farther, improving gene flow.

On average, pollinators deposited 110 pollen grains after one visit. However, considering the physical and nutritional constraints imposed by the style in this species,

only a reduced number of pollen tubes are able to grow through the pistil (Chapter 2.2). Therefore, it was clear that pollinators deposited a larger number of pollen grains than necessary, leading to high rates of post-deposition pollen losses (Inouye *et al.* 1994). These pollen losses were due to incompatible pollen deposition [self-pollen deposition after pollinators visit, as a result of secondary pollen presentation (Chapter 3.1), and/or inbred crosses] and to pollen tube competition to reach the stylar channel during the post-pollination process of germination (Chapter 2.2) (outcompeted pollen tubes, *sensu* Inouye *et al.* 1994). Thus, the quality of pollen grains and the levels of self-interference that resulted from each specific pollinator interaction will probably be the most important factors guiding female fitness, with detrimental costs over male fitness. From the male perspective, the intrinsic characteristics of the flowers and pollinators behaviour lead to the loss of a large proportion of male gametes.

Spatial and temporal variability were observed in the floral visitor's assemblage and abundance in *P. vayredae*. This clearly affected the production of offspring and thus, the introduction of new individuals into the populations each year. While in Serrat dels Boixos and Colldecarrera at least one legitimate pollinator species (involving species with apparently similar behaviours) could be found regularly visiting the flowers of *P. vayredae*, in Montmajor, the visitation rates of pollinators were always extremely low (and similar between nectarivorous and pollinivorous species). Spatial differences in insect's assemblage and abundance may arise from the microhabitat selection by pollinators, which include variation in food resources, plant traits and/or microclimatic aspects, among others (Herrera 1988, 2002; Navarro 2000; Herrera *et al.* 2002a). Furthermore, competition with other plant species may also play a major role in plant-animal interactions, as it has been observed in some pollinator attractive exotic species (Jakobsson *et al.* 2007). In Montmajor population the particular environmental conditions (exposed and windy population located in a rocky face at higher altitude) seemed to particularly affect the pollinator's occurrence, abundance and behaviour. Also, the delay in the flowering period of *P. vayredae* in this population, resulted in a broaden resource availability, as other plants started to flower in the meantime, probably leading to a higher competition for pollinators. In the remaining two populations, there seems to be only a constraint on the diversity of pollinator species, as there was one predominant species each year that usually presents reasonable visitation rates and interactions with *P. vayredae* flowers. Also, the early flowering of *P. vayredae* in these populations makes it the main species flowering and thus the major food resource. Temporal variation in insect abundance and diversity was also evident along the three years, with 2005 being the year

with the larger range of visitor species and higher abundance of legitimate pollinators, and 2006 and 2007 the years with the poorer visitor's assemblage and the lowest visitation rates (despite of the high abundance of *B. terrestris* during 2007). Climatic differences between the surveyed years may be involved in the observed temporal variation, as 2006 and 2007 presented particularly dry seasons, whereas in 2005 the usual rates of precipitation were registered (data from the *Servei Meteorològic de Catalunya*).

The high spatial and temporal heterogeneity found in the abundance of *P. vayredae* visitors may suggest that, in this species, the process of generalization would be favoured instead of specialization. However, considering the highly specialized morphology of the corolla, the number of species involved in the pollination of this plant is reduced, with the main pollinators being restricted to long-tongued and robust insects. Taking into account that these insects are generalists, exploiting other resources along their lifespan, plant-pollinator interactions in *P. vayredae* point to an asymmetric specialization. Recent studies suggest that this type of specialization is common in plant-pollinator interactions web, occurring more frequently than expected (Bascompte *et al.* 2003; Vázquez and Aizen 2004; and references therein).

Pollen limitation has been previously reported in *P. vayredae* (Chapter 2.1). The present study clearly shows that one of the major factors leading to pollen limitation is the scarcity, unpredictability and/or behavioural patterns of efficient pollinators. As a consequence, fruit and seed production were significantly affected, especially on the populations where the efficient pollinators were scarcer, as in Montmajor. Pollen limitation has been described as a common feature among animal pollinated species and cited as being more extensively present than what was previously thought (Burd, 1994, Johnson and Bond 1997, Larson and Barrett 2000; Ashman *et al.* 2004, Knight *et al.* 2005). These studies suggest that insufficient pollen receipt may commonly compromise seed production in plant populations. For example, a recent survey analysed the results of fruit production in 482 studies, and concluded that over 63% of the studied species exhibit pollen limitation at some sites, or during some years (Knight *et al.* 2005). Nonetheless, these studies usually refer to the “quantitative” component of pollen limitation (*i.e.*, stigmas receiving fewer pollen grains than the necessary to optimal ovule fertilization). Recently, Aizen and Harder (2007) made a differentiation between the “quantity” and “quality” components of pollen limitation, suggesting that studies on pollen limitation must focus on both factors. In the present study, it was observed that the quantitative component of pollen limitation was the most important factor, as only a small proportion of the available flowers received pollen. As pollinators delivered larger amounts of pollen in stigmas than

necessary to fecundate the two ovules, the qualitative component *sensu* Aizen and Harder (2007) apparently is not the most important factor of pollen limitation in this species, despite it has been also proposed to play a major role in seed production (Chapter 2.1 and 2.2). It is also worth noticing that the qualitative and quantitative components of pollinator efficiency also determined the pollen limitation. Pollinators able to deposit fewer pollen grains and visit a larger number of flowers could be involved in plant fitness improvement. Furthermore, in addition to the pollinator's services, fruit and seed production can also be influenced by other biotic and abiotic factors (*e.g.*, Casper and Niesenbaum 1993). Altogether, these factors should help to fully understand the patterns of female fitness observed in each population of *P. vayredae*.

The present study clearly revealed spatial and temporal variability in the floral visitors of *P. vayredae*. Furthermore, a large assemblage of floral visitors, mainly composed by larcenists and by a few species involved in legitimate pollination, was observed. As a consequence, the reproductive outcome was low and significantly variable among populations and years. These observations clearly reveal the need for studies under a spatial and temporal scale in order to assess the pollinator's spectrum of a plant species and further evaluate plant-animal interactions. This type of studies will provide relevant know-how on plant pollination ecology, which is important to define conservation strategies, as well as, to understand its floral evolution. In the particular case of the narrow endemic *P. vayredae*, pollen limitation and pollinator's unpredictability (both in number of species and abundance) is overcome by its perennial habit. Nonetheless, significant consequences on plant population structure and dynamics could result from its poor reproductive outcome. For all these reasons, the implementation of management strategies for the conservation of this species must necessarily contemplate efforts to protect their native pollinator fauna.

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**Appendix 4.2.1** Number of interactions of *Polygala vayredae* floral visitors in the three studied populations during 2005, 2006 and 2007. Values are given as total number of flower visits followed by the proportion of the total floral interactions (in %), given in parenthesis. The floral visitors were characterized according with the type of visit as follows: L - legitimate visitor, L<sub>i</sub> - legitimate inefficient visitor, R - nectar robber, SR - secondary nectar robber and T - nectar thief.

Visitor species	Type of visit	Montmajor			Serrat dels Boixos			Coldecarrera		
		2005	2006	2007	2005	2006	2007	2005	2006	2007
Order Hymenoptera										
<i>Anthophora</i> sp. (Anthophoridae)	L		4 (0.5)		277 (13.0)		564 (10.1)	57 (1.4)	8 (0.9)	111 (2.4)
<i>Bombus terrestris</i> (Apidae)	R	724 (33.6)	466 (56.1)	421 (90.1)	603 (28.4)	712 (73.7)	4690 (83.7)	1903 (45.8)	620 (69.1)	3883 (84.5)
<i>Bombus pascuorum</i> ,queen (Apidae)	L	87 (4.0)	51 (6.1)		964 (45.3)	164 (17.4)	160 (2.9)	1778 (42.8)	162 (18.1)	294 (6.4)
<i>Bombus lucorum</i> (Apidae)	L				107 (5.0)					
<i>Bombus pratorum</i> (Apidae)	R		42 (5.1)		8 (0.4)	56 (5.8)		156 (3.8)	61 (6.8)	
<i>Bombus lapidarius</i> (Apidae)	R		3 (0.4)							3 (<0.1)
<i>Bombus hortorum</i> (Apidae)	R						18 (0.3)			
<i>Psithyrus</i> sp. (Apidae)	R									4 (<0.1)
<i>Apis mellifera</i> (Apidae)	T	145 (6.7)		8 (1.7)	3 (0.1)					119 (2.6)
<i>Eucera longicornis</i> (Apidae)	L							19 (0.5)		10 (0.2)
<i>Halictus</i> sp. (Halictidae)	L	52 (2.4)		2 (0.4)	40 (1.8)					
<i>Osmia rufa</i> (Megachilidae)	T		14 (1.7)		5 (0.2)			31 (0.7)		12 (0.3)
<i>Xylocopa violacea</i> (Xylocopidae)	R							6 (0.1)		
<i>Formica gagates</i> (Formicidae)	SR	5 (0.2)	1 (0.1)			4 (0.4)		2 (<0.1)	10 (1.1)	4 (<0.1)
<i>Temnothorax</i> sp. (Formicidae)	SR	2 (0.1)	4 (0.5)			12 (1.2)			1 (0.1)	3 (<0.1)

<i>Myrmica</i> sp. (Formicidae)	SR		3 (0.4)					1 (0.1)	
Unknown (Formicidae)	SR	111 (5.2)	18 (2.2)						1 (<0.1)
Order Diptera									
<i>Bombylius</i> sp. (Bombyliidae)	L <sub>i</sub>	7 (0.3)	12 (1.4)	8 (1.7)		44 (0.8)	5 (0.1)	2 (0.2)	13 (0.3)
Order Lepidoptera									
<i>Macroglossum stellatarum</i> (Sphingidae)	L <sub>i</sub>	821 (38.2)	166 (20.0)	28 (6.0)	14 (1.4)	32 (0.6)	49 (1.2)	32 (3.6)	
<i>Hemaris fuciformis</i> (Sphingidae)	L <sub>i</sub>	217 (10.1)			41 (1.9)	8 (0.1)	116 (2.8)		99 (2.2)
<i>Gonepteryx rhamni</i> (Pieridae)	L <sub>i</sub>	6 (0.3)			66 (3.1)	68 (1.2)	13 (0.3)		11 (0.2)
<i>Gonepteryx cleopatra</i> (Pieridae)	L <sub>i</sub>				6 (0.3)	18 (0.3)	4 (0.1)		6 (0.1)
<i>Leptidea sinapis</i> (Pieridae)	T								5 (0.1)
<i>Papilio machaon</i> (Papilionidae)	T						6 (0.1)		14 (0.3)
<i>Pyronia</i> sp. (Nymphalidae)	T	2 (0.1)			6 (0.3)		17 (0.4)		
<i>Aglais urticae</i> (Nymphalidae)	L <sub>i</sub>		1 (0.1)						
<i>Vanessa atalanta</i> (Nymphalidae)	L <sub>i</sub>	2 (0.1)	7 (0.8)						
<i>Vanessa cardui</i> (Nymphalidae)	L <sub>i</sub>		38 (4.6)						
<b>Total no. of visits</b>		<b>2181</b>	<b>830</b>	<b>467</b>	<b>2126</b>	<b>966</b>	<b>5602</b>	<b>4162</b>	<b>897</b>

Appendix 4.2.1 (cont.)



## 4.3 Consequences of nectar robbing in the fitness of a threatened plant species

Chapter section submitted as an original article to SCI journal:

Castro S, Silveira P, Navarro L. 2007. Consequences of nectar robbing in the fitness of a threatened plant species. *Plant Ecology* (submitted).





## Abstract

The effect of nectar robbing on plant fitness is poorly understood and restricted to a few plant species. Furthermore, the available studies generally evaluate the effects of nectar robbing over female fitness disregarding the male component. Here we measured the effects of the nectar-robbing bumblebees on male (measured as pollen analogue flow distance) and female reproductive success (measured as seed production) in the insect-dependent *Polygala vayredae*, a narrow endemic species from pre-Pyrenees (Spain). Intense nectar robbing by bumblebees significantly reduced the available nectar to legitimate pollinators in the studied population, and this reduction affected both to male and female fitness. Significant differences were observed in fluorescent dye dispersion between robbed and non robbed flowers within the population. Fluorescent dyes from non robbed flowers were dispersed to larger distances and over a larger number of flowers when compared with robbed ones. Moreover, significant differences were observed both in fruit set and seed ovule ratio between the two groups, with non robbed flowers presenting higher reproductive outcomes. However, no effect on seed weight was detected among treatments. The data obtained suggest that, in this species, nectar robbing has important indirect and negative effects on plant fecundity, through both male and female functions, due to a modification in the foraging behaviour of legitimate visitors.

**Keywords** bumblebees; female fitness; foraging behaviour; male fitness; nectar robber; reproductive success

## **Introduction**

Plants that provide floral rewards to attract pollinators frequently attract a wide range of other floral visitors. Among them are nectar robbers that, when facing long and narrow corollas, or closed ones, typically make an incision at the base of the tube to reach nectar, without entering in contact with reproductive structures (Inouye 1980). Nectar robbers can directly or indirectly affect plants and its interactions with legitimate visitors. Several studies have already shown direct impacts via removal of floral resources, flower damaging or shortening of flower lifespan (*e.g.*, Traveset *et al.* 1998; Navarro 2001; González-Gómez and Valdivia 2005; Zhang *et al.* 2007), and indirect impacts as territorial behaviour of nectar robbers (*e.g.*, Roubik 1982) or changes in the patterns of available nectar, which affect legitimate pollinator's foraging behaviour (*e.g.*, Irwin and Brody 1998). For example, intense nectar robbing or nectar thieving can indirectly affect legitimate pollinator's behaviour by increasing foraging flight distances (*e.g.*, Zimmerman and Cook 1985; Maloof 2001), reducing the number of visited flowers per patch or inflorescence (*e.g.*, Hodges 1985; Irwin and Brody 1998; González-Gómez and Valdivia 2005) or reducing the time spent per flower (*e.g.*, Thomson and Plowright 1980; Zimmerman and Cook 1985). Consequently, these changes in pollen flow distances and outcrossing rates or alterations in visit's efficiency and pollinator's fidelity, will certainly affect the plant reproductive outcome. Although in some cases nectar robbers may act as direct mutualists (*e.g.*, Higashi *et al.* 1988; Navarro *et al.* 1993; Navarro 2000) or even present a neutral effect in plant reproduction (*e.g.*, Arizmendi *et al.* 1996; Stout *et al.* 2000), most of the accumulated evidences indicate direct or indirect negative effects in plant fitness (*e.g.*, Roubik 1982; Traveset *et al.* 1998; Irwin and Brody 1999; Irwin *et al.* 2001; Zhang *et al.* 2007). From this, it is clear that the effect of nectar robbing on the plant reproductive success result from a complex and variable array of positive, neutral or negative interactions rather than the simplistic single side-effect scenario (Maloof and Inouye 2000; Irwin *et al.* 2001).

From the plant perspective, most studies on nectar robbing have only considered the effects of nectar robbers on the maternal plant function (Maloof and Inouye 2000; for a review see Irwin *et al.* 2001). However, in hermaphroditic plants the reproductive success is the sum of the gains obtained through both male and female functions. Because seed production is often pollen and/or resources limited, pollinator visitation behaviour may more strongly affect the seeds sired than the seeds produced (*e.g.*, Young and Stanton 1990; Irwin and Brody 2000; and references therein); therefore, to accurately assess the effects of nectar robbing on plant fitness, both male and female function should be

considered. Despite of the impact of nectar robbing or nectar thieving on plant fecundity has already been assessed in various tropical and temperate plant species, there are only few studies that examined both aspects of male and female reproductive outcome in relation to nectar larceny (Wyatt 1980; Fritz and Morse 1981; Morris 1996; Irwin and Brody 1999; Zhang *et al.* 2007).

*Polygala vayredae* Costa (Polygalaceae) is a narrow endemic species from oriental pre-Pyrenees (Spain), with long closed corolla tubes and nectar rewards located at the base of the floral tubes. Despite the flowers of *P. vayredae* receive visits from more than 24 floral visitors, this species frequently suffers pollen limitation due to pollinator scarcity, with just two species of long-tongued bees (*Anthophora* sp. and the queens of the bumblebee *Bombus pascuorum*) and two pollinivorous (*Eucera longicornis* and *Halictus* sp.) behaving as legitimate pollinators. Only these species are able to move the keel downwards and enter in contact with the reproductive structures when probing for nectar or collecting pollen. The remaining species act as nectar robbers, secondary nectar robbers or nectar thieves (*sensu* Inouye 1980). A set of six different species of short-tongued bumblebees (from which *B. terrestris* is the most frequent) were active nectar robbers accounting for 64.2% of the total visits during the period of 2005-2007 (Chapter 4.2). In fact, the frequency of nectar robbing assessed during this period was always above 80% (Chapter 4.1 and S. Castro, personal observations). Furthermore, due to the closed structure of the flower, nectar robbers do not enter in contact with the reproductive structures and, thus, never accidentally pollinate the plant. Also, no significant floral damages induced by nectar robbers have been observed (Chapter 2.1).

The knowledge of these intense (robbers) and crucial (pollinators) plant-animal interactions occurring in *P. vayredae* led us to consider whether nectar robbing would indirectly affect the reproductive outcome of this species. To address this question the effect of nectar robbing on male and female fitness was experimentally measured, through pollen analogues flow distance and seed production, respectively. The effect of nectar robbing on available nectar was also estimated. The information gathered constitutes the first report on nectar robbing effects on *Polygala* genus and increments the available information needed to understand the striking consequences of nectar robbing on plant fitness and plant-animal interactions.

## Materials and methods

### *Plant and study area*

*Polygala vayredae* is a narrow endemic species restricted to an area of approximately 12 km<sup>2</sup> in Alta Garrotxa, Girona (Catalunya, Spain), where it occurs in few dense populations. According with the IUCN categories, a preliminary endangered status has been given to this plant (vulnerable; VV.AA. 2000). This species is a small shrublet, annually sending up shoots from a rootstock forming dense clusters of several individuals. It presents large, self-incompatible papilionate flowers, with the two lateral petals forming a long tube ( $14.5 \pm 0.78$  mm long; for illustrations see Chapter 2.1). Nectar is produced at the base of the corolla (daily nectar production:  $1.9 \pm 0.86$  µl with  $16.0 \pm 6.23\%$  of sugar) and flowers are able to re-synthesise after several removals (Chapter 2.1). Peculiarly, flowers subjected to intense nectar robbing produce less amount of nectar (in volume and amount of sugar) than non robbed flowers (S. Castro, unpublished data). Furthermore, flowers possess a mechanism of secondary pollen presentation where the pollen is presented to the pollinators in a specialized region of the stigma (the pollen presenter; Chapter 3.1).

The present study was carried out during the spring of 2007 (flowering peak of *P. vayredae*) in the population of Coldecarrera at the natural protected area of Alta Garrotxa (UTM DG57). This population occurs in mesophytic and xeric meadows (*Mesobromion*) with *Pinus sylvestris* and *Buxus sempervirens*, under the *Quercetum pubescentis* domain. It was selected due to its accessibility and pollinator's availability.

### *Nectar availability*

The amount of available nectar of robbed and non robbed flowers was assessed at the first hours of the day (usually between 0800 and 1000 h, GMT) in 115 randomly selected flowers along the population. Nectar production was measured with capillary micropipettes and sugar concentration was determined (in w/w, %) using a portable refractometer. The amount of sugar produced by each flower was calculated following Prys-Jones and Corbet (1987).

### *Effect of nectar robbing on pollen flow*

To evaluate the effect of nectar robbing on male fitness (pollen dispersion), fluorescent powdered dyes were used as pollen analogues (Waser and Price 1982). It has been previously observed that dye transfer closely resembles pollen transfer when bumblebees are the pollen vector (*e.g.*, Waser 1988; Rademaker *et al.* 1997; Adler and Irwin 2006), despite of the different dispersal properties of the dye and pollen grains (Thomson *et al.*

1986). A 0.25 m<sup>2</sup> cluster with flower buds was protected with a mosquito net to avoid nectar robbing. When flowers opened, the mosquito net was removed and two sets of flowers were prepared: 1) non robbed flowers - fluorescent dye was applied on the pollen presenter of 30 newly open flowers protected at the base of the corolla using transparent tape, to avoid nectar robbing; 2) robbed flowers - fluorescent dye of a different colour was applied on the pollen presenter of 30 newly open flowers, daily subjected to nectar robbing. Nectar robbing was performed by cutting a hole at the base of the corolla and removing the available nectar daily with micro-capillary tubes. Three replicates separated by intervals of over 100 m were set within the population. Furthermore, in the intermediate replica different coloured dyes were used to avoid erroneous results. After eight days, up to 50 flower samples were collected at several distances from each cluster source (1, 2, 3, 4, 5, 10, 25-50, 50-100 m) and preserved at -4 °C. At the laboratory, flowers were examined under UV light using a stereo binocular microscope. The presence/absence of each dye powder grains deposited on the stigma was recorded for each distance.

#### *Effect of nectar robbing on fruit production and seed weight*

To evaluate the effect of nectar robbing on female fitness (fruit and seed production), 500 flower buds were randomly selected along the population and the following treatments were applied: 1) flower buds were protected from nectar robbing; 2) flower buds were subjected to daily nectar robbing after anthesis (accordingly with the procedure described above); and 3) unmanipulated flowers were left for open visitation as a control of the population. Flowers were open pollinated and fruit and seed production were recorded when mature. Seeds were collected to determine seed weight. For that, at the laboratory, seeds were dehydrated under natural conditions, maintained in a vacuum excicator with silica gel for 24 h and weighed in an analytical balance (0.01 mg precision).

#### *Statistical analysis*

The differences in nectar volume and sugar amount between robbed and non robbed flowers were analysed with a Mann-Whitney rank sum test, while nectar concentration was analysed with a *t* test. The proportions of robbed and non robbed flowers without nectar reward were analysed with a *z* test.

The proportions of flowers with fluorescent dye for each distance (categorical data) were adjusted to a binomial distribution and analyzed with Generalized Linear/Nonlinear Model with a logit link function. Type 3 Likelihood-Ratio test was computed. The

proportions of flowers receiving fluorescent dye from robbed and non robbed flowers were analysed with a z test.

The effect of nectar robbing on fruit set and seed ovule ratio was evaluated with a  $\chi^2$  test for comparison of more than two proportions and multiple comparison tests for proportions were made according with Zar (1984). Differences among treatments in seed weight were analyzed with a one-way ANOVA. The Generalized Linear Model procedure was followed due to unbalance data.

## Results

### Nectar availability

Robbed and non robbed flowers of *P. vayredae* presented different nectar rewards (Table 4.3.1). The nectar volume encountered in non robbed flowers was significantly higher than the one found in robbed flowers ( $T = 1,593.5$ ,  $P = 0.001$ ). Furthermore, despite no significant differences were observed in the mean nectar concentration ( $t = 0.24$ ,  $P = 0.812$ ), the mean amount of sugar (*i.e.*, energy) collected in non robbed flowers was significantly higher ( $T = 1,552.0$ ,  $P = 0.004$ ). Moreover, intense nectar robbery seemed to decrease significantly the ability of the flower to re-synthesise nectar ( $z = 2.18$ ,  $P = 0.029$ ), as a high proportion of robbed flowers stopped nectar production, losing one important pollinator attractive feature (Table 4.3.1).

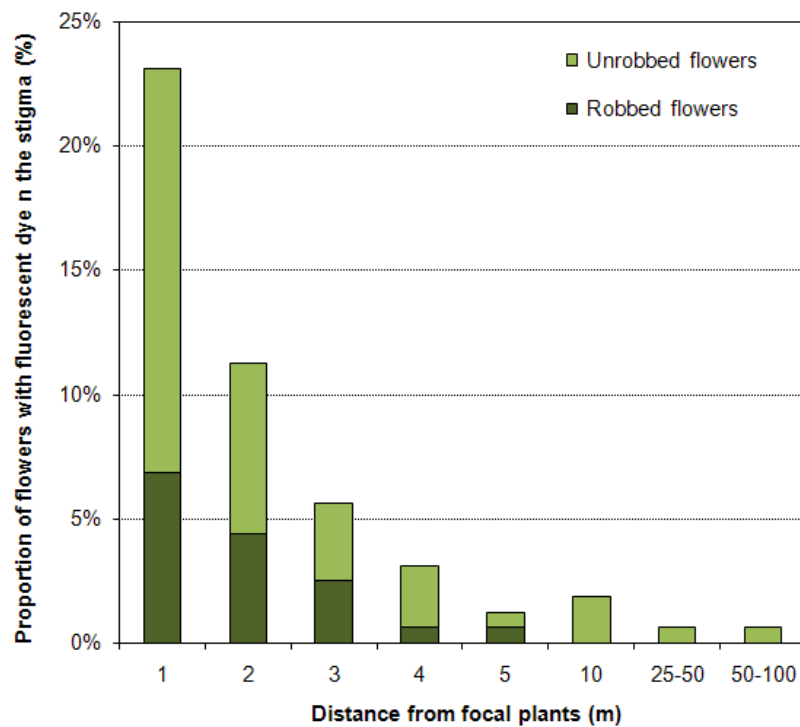
**Table 4.3.1** Nectar available in robbed and non robbed flowers of *Polygala vayredae* in Coldecarrera population during spring of 2007.

Treatment	n	Volume ( $\mu$ l)	Nectar concentration (%)	Sugar amount (mg)	Proportion of rewardless flowers (%)
Robbed flowers	95	$0.34 \pm 0.772$	$70.2 \pm 19.99$	$0.15 \pm 0.266$	44.2
Non robbed flowers	20	$0.67 \pm 0.597$	$71.5 \pm 23.41$	$0.33 \pm 0.322$	15.0
Comparison test		$T = 1,593.5^{***}$	$t = 0.24$ n.s.	$T = 1,552.0^{**}$	$z = 2.18^*$

Notes: Volume, nectar concentration and sugar amount are given as mean and standard deviation of the mean; for the calculation of mean nectar concentration, rewardless flowers were excluded. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*  $P < 0.001$ , n.s. – not significant.

*Effect of nectar robbing on pollen flow*

Both robbed and non robbed flowers of *P. vayredae* were able to disperse the pollen. However, a significantly higher pollen flow ( $z = 3.14$ ,  $P = 0.002$ ) was observed in non robbed flowers (4.1%) in comparison with robbed ones (1.9%). In both cases, pollen flow was found to be higher in the first meters, drastically reducing as the distance to the focal clusters increased ( $\chi^2 = 68.11$ ,  $P < 0.001$  and  $\chi^2 = 42.49$ ,  $P < 0.001$  for non robbed and robbed flowers, respectively; Fig. 4.3.1).



**Fig. 4.3.1** Fluorescent dyes dispersion among robbed (dark green) and unrobbed flowers (light green) of *Polygala vayredae*: proportions of flowers with fluorescent dye in the stigma represented in function of the distance to the cluster of focal plants.

*Effect of nectar robbing on fruit production and seed weight*

Fruit set and seed ovule ratio were significantly affected by nectar robbing ( $\chi^2 = 6.94$ ,  $P < 0.05$  and  $\chi^2 = 23.39$ ,  $P < 0.001$ , respectively; Table 4.3.2). Non robbed flowers and control flowers produced significantly more fruits and seeds than robbed flowers ( $P < 0.05$ ). Concerning seed weight, no significant differences were observed among treatments ( $F = 1.83$ ,  $P = 0.163$ ).

**Table 4.3.2** Fruit set, seed ovule ratio and seed weight in open pollinated flowers of *Polygala vayredae* subjected and not subjected to nectar robbing.

Treatment	n	Fruit set (%)	Seed ovule ratio (%)	Seed weight (mg)
Robbed flowers	200	15.5 <sup>a</sup>	12.3 <sup>a</sup>	8.23 ± 1.425
Non robbed flowers	200	25.0 <sup>b</sup>	18.8 <sup>b</sup>	7.86 ± 1.099
Control	100	26.0 <sup>b</sup>	17.5 <sup>b</sup>	8.27 ± 1.396
Comparison test		$\chi^2 = 6.94^*$	$\chi^2 = 23.39^{***}$	$F = 1.83$ n.s.

Notes: Fruit set and seed ovule ratios are given as percentages and seed weight is given as mean and standard deviation of the mean; data were collected in Coldecarrera population during 2007; different letters reveal significant differences. \* $P < 0.05$ , \*\*\*  $P < 0.001$ , n.s. – not significant.

## Discussion

Nectar robbing is an ecological interaction widely distributed in temperate and tropical regions, with bumblebees being common nectar robbers of many flower species (Inouye 1983). As nectar robbers can affect the interaction among plants and its legitimate pollinators, they cannot be disregarded when studying the fitness of flowering plants. However, due to the scarcity of available information, the actual role of nectar robbing on plant-pollinator interactions and its impacts on the reproductive outcome are largely unknown. The present study provides the first report on the consequences of nectar robbing in the reproductive success within *Polygala* genus, with further information given on nectar robbing in *P. vayredae*.

Nectar robbing can deeply influence the patterns of nectar availability and this change may affect flower attractiveness and floral visitor's behaviour (e.g., Zimmerman and Cook 1985; Irwin and Brody 1998; Irwin *et al.* 2001; Maloof 2001; González-Gómez and Valdivia 2005). Previous observations indicated that nectar robbers were steady components of *P. vayredae* flower-visitor interactions (Chapters 4.1. and 4.2). In the present study it has been showed that they affected the nectar availability in the studied population during spring of 2007: when visiting robbed flowers, floral visitors obtained less nectar rewards and, consequently, less energy; furthermore, flower visitors frequently encountered rewardless flowers (44%). These observations suggest variable and unpredictable nectar availability in the population as a consequence of nectar robbing. A reduction in nectar availability or changes in nectar production patterns as a result of nectar robbers exploitation have been observed in several other species (e.g., *Mertensia paniculata* L., Morris 1996; *Macleania bullata* Yeo, Navarro 1999; *Puya coerulea* Miers,



González-Gómez and Valdivia 2005; *Moussonia deppeana* Klotzsch ex Hanst., Ornelas *et al.* 2007), with subsequent impacts in pollinator's visitation for some of them. Furthermore, in several species, the lack of nectar may result from damages inflicted by the nectar robber on the flower and nectar gland (*e.g.*, Traveset *et al.* 1998; González-Gómez and Valdivia 2005). This was not the case of *P. vayredae* where, despite some damages were caused to the corolla, the reduction in available nectar was the result of successive nectar extractions, with no harm being inflicted to the nectar gland (Chapter 2.1).

In *P. vayredae* pollen dispersal appears to be negatively affected by nectar robbing, as high levels of nectar robbing reduced the dispersal distance of fluorescent dye within the studied population. Furthermore, as pollen analogues from robbed flowers were not found in far distances from the cluster source, it seems that the lack/low resources of these flowers, lead the pollinators to change their behaviour and leave the population. On the other hand, pollen from non robbed flowers was dispersed far away within the population, despite a drastic decrease in the number of visited flowers was observed as the distance from the cluster source increased. Although the consequences of nectar larceny on male function have been examined in few plant species, different effects have been observed. While in *M. paniculata* nectar-robbing bumblebees had a positive effect on pollen removal in young flowers but no impact in the final male fitness of the flower (Morris 1996), in the *Asclepias syriaca* L. no effects of nectar-thieving ants on pollinia removal were observed (Fritz and Morse 1981). On the other hand, in *Ipomopsis aggregata* (Pursh) V.E. Grant, nectar-robbing bumblebees considerably decreased pollen donation, negatively affecting the male function (Irwin and Brody 1998). To some extent, nectar robbing has been regarded as a positive interaction when the change in pollinator's behaviour results in an increase in the distance between plant flights, improving pollen dispersal and thus gene flow (*e.g.*, Zimmerman and Cook 1985; Maloof 2001), or in a decrease in the visitation rate within inflorescences or plants, reducing geitonogamous pollination (*e.g.*, Hodges 1985; Klinkhamer and de Jong 1993). However, in other cases legitimate pollinators may switch to different plant species or even depart from the population (*e.g.*, Roubik 1978, 1982). The behaviour of *Bombus pascuorum* queens, the main pollinator in the studied population (Chapter 4.2), seems in accordance with the results obtained in the pollen flow experiment, as this bumblebee is usually observed exploiting a high number of flowers within a patch, when nectar availability is high (as in the blossom beginning), and a lower number if the quantity of nectar is more variable due to high nectar robbing (as in the blossom peak). However, in both cases, when *B. pascuorum* leaves the flower clusters, it always flies away several meters, sometimes even out of sight, before starting new flower

exploitations (S. Castro, personal observations). Thus, pollinators fly further within the population after visiting non robbed flowers in search for more food rewards, and were more likely to leave the population after visiting several rewardless flowers subjected to high nectar robbing. This is only partially in accordance with the foraging theory, as while it seems that the pollinator leaves the cluster or even the population when the rate of energy intake is lower than the potential energy intake available in the habitat (Charnov 1976), an inverse relationship between nectar rewards and interplant pollinator flight distance (Pyke 1978) was not observed. However, this theory is primarily based on the assumption that pollinators had no previous knowledge of flower's reward status. The main pollinators of *P. vayredae* appear to selectively visit non robbed flowers, which may be an indication of a prior recognition of the available rewards or scent marks left by previous floral visitors (Goulson *et al.* 1998). If this is the case, predictions about pollinator's foraging behaviour could significantly be altered. Further studies monitoring different robbery frequencies, available rewards, and pollinator's visitation rates and behaviour are needed to better understand these complex plant-animal interactions.

Regarding female fitness, nectar robbing significantly decreased the number of fruits and seeds produced. On a meta-analysis study integrating the available works on nectar robbing effects, Irwin *et al.* (2001) observed that in general the floral larceny had weak but negative effects on female reproductive success. Furthermore, the authors observed that the type of larcenist and pollinators had significant impacts on the resulting fitness. For insect-pollinated plants, floral larceny by other insects had a weak positive effect. However this result was probably influenced by the fact that there are several mutualistic plant-robber interactions where the insect accidentally pollinates the flower (*e.g.*, Higashi *et al.* 1988; Navarro 2000). In *P. vayredae* as the corolla is a closed structure, nectar robbers were never involved in pollination, exerting an indirect negative effect on the realized female fitness. Similar observations were obtained, for example, in *Quassia amara* L. (Roubik *et al.* 1985), *Vitex negundo* L. (Reddy *et al.* 1992), *Ipomopsis aggregata* (Irwin and Brody 1998), and *Duranta repens* L. (L. Navarro and R. Medel, unpublished data). On the other hand, seed weight was not affected by nectar robbing. This agrees with previous observations on nectar production patterns, where intense nectar robbing also decreased the total amount of nectar produced per flower (S. Castro, unpublished data). From these results, it seems that in *P. vayredae*, nectar robbing did not affect seed weight through the energetic investment on nectar re-synthesis, contrarily to what was observed in other species (Southwick 1984; Pyke 1991).

Taken all together, the results obtained in the present study suggest an indirect negative effect of the nectar robbing on plant reproductive outcome, through a decreasing in the available nectar and consequent change in pollinator visitation behaviour that, when avoiding robbed flowers or leaving the population, reduces pollen flow and dispersal distances (male function), as well as it reduces fruit and seed production (realized female fitness). Considering the lack of studies on the cumulative effects of robbing on both male and female function, the importance of nectar robbers to plant ecology and evolution may have been underestimated in the past and should be further considered in future studies.

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## Chapter 5

### Dispersal ecology

#### 5.1 Dispersal mechanisms in the narrow endemic *Polygala vayredae* Costa (Polygalaceae): efficiency of the dispersal syndromes and spatio-temporal variations on ant dispersal assemblages

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## Abstract

The present study describes the dispersal mechanisms of *Polygala vayredae*, a narrow endemic species from pre-Pyrenees, and analyses the functioning of its dispersal syndromes (anemochory, through bicarpelar winged capsules, and myrmecochory, through seeds with ant-attractive elaiosomes), the spatio-temporal variability of disperser's assemblages, their foraging behaviour and dispersal ability, as well as, the role of the elaiosome on ants attraction and seed germination. During the process of maturation, the capsules of *P. vayredae* open its wings and expose the mature seeds before leaving the mother plant. The dispersion of diaspores occurs when (1) either the capsules or the seeds fall beneath the mother plant by the action of their weight (barochory), with no significant differences in dispersal distances being observed between diaspores, or when (2) the seeds are directly collected in the suspended capsules by ants (myrmecochory). Thus, the functional adaptation to anemochory is currently reduced, limiting the ability for long distance dispersal. The plant is essentially myrmecochorous, with a diverse array of ant species being involved in seed dispersal, and with the elaiosome playing a major role in ant's attraction. Furthermore, considering the ant's ability to disperse the seeds and the dispersal distances, several functional groups were observed. From the plant's perspective for dispersal, the majority of ant species had a positive interaction with the seeds (e.g., *Formica* sp., *Lasius* sp.). Overall, dispersal distances were limited and mainly determined by the ant's body size. Also, variation in the frequency of interactions and in the functional groups assemblage was observed at both spatial (among three populations) and temporal scales (along two consecutive years). Seed predation was not a constant occurrence, being low in any population. Finally, no significant differences were observed in the germination rates after ant- or artificially-removed elaiosome with respect to seeds with elaiosome. The ability of *P. vayredae* plants to annually produce shoots from a rootstock, linked with a dispersal mechanism that enhances seed dispersal within the population could be the main factors explaining the actual density of this species in the studied populations.

**Keywords** alate capsule; anemochory; ants; barochory; dispersal distances; elaiosome; myrmecochory; seed predation; seed germination

## Introduction

The dispersal of diaspores is an important transition step in the life cycle of seed-bearing plants, determining the dynamics of natural habitats, the structure and succession of the vegetation and the gene flow within and among populations (Gorb and Gorb 2003). So far, several plant introduction experiments revealed that species with a restricted area of distribution may present suitable habitats available for colonization (*e.g.*, Colas *et al.* 2000; Münzbergova and Herben 2004), but in many cases the low fecundity and/or reduced dispersal ability lead to an aggregate spatial distribution within the population and to a narrow distribution area (Lavergne *et al.* 2004; Imbert 2006). Therefore, the knowledge of dispersal methods is a necessary condition to understand the processes and mechanisms of species survival and to predict population dynamics.

During the process of evolution, as a result of different selective pressures exerted by various factors in diverse habitats, plants developed an array of structures adapted to particular biotic and/or abiotic dispersal vectors. Among the diversity of diaspores that can be found, the species from the genus *Polygala* L. (Polygalaceae) evolved to exhibit several distinct dispersal syndromes (*e.g.*, winged structures, elaiosomes) theoretically linked with specific dispersal mechanisms (anemochory, myrmecochory, ornithochory, diplochory and epizoochory; reviewed in Forest *et al.* 2007). In the studies performed so far in *Polygala* spp. of temperate regions, it has been shown that the diaspores presented mainly two means of dispersion (diplochory): while it is known that the seeds of several species are dispersed by ants (myrmecochory; Sernander 1906; Lack and Kay 1987; Oostermeijer 1989), the wind dispersal of the capsules prior to seed dispersal has also been observed (anemochory; Sernander 1906; Lack and Kay 1987). In myrmecochory, the seeds characteristically present an elaiosome rich in lipids that attract ants due to its chemical similarity to insect hemolymph (Lanza *et al.* 1992; Hughes *et al.* 1994). This interaction has been described as mutualistic, with the ants carrying the seeds to their nests and benefiting from the nutritive resources provided by the elaiosome (Beattie 1985; Morales and Heithaus 1998; Wolff and Debussche 1999), and the plant profiting from the dispersal services of their seeds (van der Pijl 1982; Beattie 1985). As a consequence, associated with the direct benefit of dispersal, plants may also attain selective advantages through other ways, such as: predator avoidance (*e.g.*, Heithaus 1981; Boyd 2001; Manzaneda *et al.* 2005; and reviewed in Giladi 2006), reduction in parent-offspring conflict and sibling competition (*e.g.*, Handel 1978; despite seed relocation in clusters inside the nests), relocation of the seeds to more favourable sites for germination and establishment (*e.g.*, nutrient enriched, moistly environment; Culver and Beattie 1978; Oostermeijer 1989;

Eldridge and Myers 1998; but see Horvitz and Schemske 1986a), seed manipulation by removal of the elaiosome with positive consequences on seed germination (*e.g.*, Culver and Beattie 1980; Horvitz and Beattie 1980; Gómez *et al.* 2003) and/or even fire avoidance (*e.g.*, Hughes and Westoby 1992a). On the other hand, in anemochory, the diaspores present structural adaptations (such as membranaceous wings or pappus) that will potentially increase their dispersal distances, leading to a higher probability to colonize new places and promoting gene flow. Nonetheless, despite the opportunity for long-distance dispersal, this mechanism is also subjected to high levels of uncertainty associated with the aerial transportation processes, and thus it is less reliable when compared with biotic vectors.

Spatial and temporal variation in the composition, visitation rates and dispersal behaviour of visitor's assemblage may result in different selection patterns on the plants. Up to this moment, a number of studies in myrmecochorous plants have evidenced the importance of such variations on the successive stages of the dispersal cycle, such as seed predation, seed removal and dispersal distances, seed germination, and/or seedling survival (*e.g.*, Pudlo *et al.* 1980; Horvitz and Schemske 1986b; Gorb and Gorb 1999; Garrido *et al.* 2002; Gómez *et al.* 2003; Manzaneda *et al.* 2005). Despite many studies of spatial variation in a local framework (most of them mentioned above) and recently even at a broad multipopulation scale (*e.g.*, Guitián *et al.* 2003; Rey and Manzaneda 2007) have been performed so far, the analysis of temporal variation in ant dispersers assemblage has remained largely neglected (but see Manzaneda *et al.* 2007). The analysis of the consequences of both sources of variation will potentially enable the perception of evolutionary patterns of specialization within plant communities, particularly the evolution of mutualisms.

The aim of the present study was to document the mechanisms of diaspore dispersal in the narrow endemic species *Polygala vayredae* Costa. This species occurs in the oriental pre-Pyrenees and presents a restricted distribution area of approximately 12 km<sup>2</sup>, being described as a relic that survived through the Quaternary glaciations in the enclave where it actually occurs (Bolós 1946). In the few existing populations, the plant forms dense carpets of several hardly distinguishable individuals. As it is characteristic of other species from the genus, *P. vayredae* presents two structurally different diaspores, associated with two dispersal syndromes: bicarpelar winged capsules (anemochory) and seeds with an ant-attractive elaiosome (myrmecochory). However, no focused studies on the functional aspects of these structures have been performed so far, and thus, no data is available on the quantitative importance of each dispersal syndrome. In the present study we

evaluated the efficiency of each dispersal syndrome and the temporal and spatial variation in the seed dispersal mechanisms of *P. vayredae*. For this, primary and secondary dispersal experiments were performed in three populations during three years to determine the dispersal vectors, the effective dispersal distances and the function of the elaiosome in disperser's attraction. Ant's assemblage and behaviour was also documented along time and space, together with seed predation and the effect of elaiosome removal by ants on seed germination.

## Materials and methods

### *Plant and study area*

*Polygala vayredae* is a small perennial shrub distributed in a restricted area in Alta Garrotxa, Girona (Catalunya, Spain). This narrow endemic species occurs in a few dense populations in mesophytic and xeric meadows (*Mesobromion*), with *Pinus sylvestris* and *Buxus sempervirens*, and in rocky places (*Saxifragion mediae*), mainly under the *Quercetum pubescentis* domain. New shoots are produced each year from a rootstock, resulting in dense clusters of hardly distinguishable individuals. One-year old ramets produce small axilar inflorescences of 1-3 flowers in early spring (April to May), with each flower having a bilocular ovary with one ovule per locule. Fruiting occurs in late spring (from May to June) with the production of a dehiscent capsule containing two seeds. The capsules are large ( $10.0 \pm 1.04$  mm long and  $8.8 \pm 0.79$  mm wide), winged ( $1.8 \pm 0.27$  mm wide) and presented in a  $6.3 \pm 0.86$  mm pedicel in a variable angle with the ramet ( $104.8 \pm 49.13^\circ$ ). The hairy seeds ( $4.7 \pm 0.24$  mm long and  $2.3 \pm 0.09$  mm wide) present a large external elaiosome ( $3.0 \pm 0.44$  mm long) and a weight of  $9.1 \pm 1.62$  mg (dry weight). The seeds germinate at the beginning of spring of the following year, being nearly absent from the soil seed bank of subsequent years.

The present study was performed in the natural protected area of Alta Garrotxa (UTM DG57 and DG58) at three populations (Montmajor, Serrat dels Boixos and Coldecarrera; for details see Chapter 2.1 and Fig. 1.1) during the fructification period of three consecutive years (2004-2006). The study sites were selected to include different types of habitats within the most representative populations of *P. vayredae*.

### *Primary dispersal*

Primary seed dispersal was evaluated in all populations on three consecutive years. Forty ramets of *P. vayredae* with a mature fruit primed for dehiscence were randomly selected in every population. A 60 cm diameter aluminium tray with a circular hole at the centre (to

fit around the ramet) was mounted below each ramet. Trays with such width were used since preliminary observations indicated that the distances of primary dispersal were usually less than 40 cm. The aluminium plates were then densely coated with glue to ensure that the fallen diaspores remain adhered to the tray. The type of diaspore (*i.e.*, capsule or seed) and respective distance from the mother plant (primary seed dispersal) were recorded in each tray.

#### *Secondary dispersal and seed predation*

Secondary seed dispersal and seed predation (with rodents being the major seed predators) were investigated using selective exclusion experiments (“cafeteria” method) with four treatments: 1) *Free exposure to dispersers and predators*, 10 seeds were placed over a Petri dish; 2) *Rodents exclusion*, 10 seeds were placed over a Petri dish protected by a metallic cage, allowing the access to ants but not to rodents; 3) *Ants exclusion*, 10 seeds were placed over a Petri dish with the borders imbued with glue to prevent the entry of ants but allowing the access to rodents; 4) *Ants and rodents exclusion* (control group), 10 seeds were placed over a Petri dish with glued borders and protected by a metallic cage (*i.e.*, the combination of treatments 2 and 3). Furthermore, to evaluate the role of the elaiosome in disperser’s attraction, the treatments were duplicated in two sets, one containing seeds with fresh elaiosomes, and another with seeds where the elaiosome was removed after drying. Each Petri dish of the two sets of treatments was randomly spaced over a circle of approximately 3.14 m<sup>2</sup>, and maintained for a total of three days. During this period, the number of seeds that had been removed from each treatment was recorded every 24 h. Also, the removed seeds were replaced by fresh ones so that the total number of 10 seeds was offered each 24 h. These experiments were performed altogether in three different sites of each population, simultaneously in all populations and in the three consecutive years.

#### *Disperser’s assemblage, frequency of interaction and foraging behaviour*

The disperser’s assemblage and foraging behaviour were assessed by direct observations of dehiscent mature fruits during two consecutive years (2005 and 2006) in the three populations. The observations were made in several randomly selected areas of approximately 1 m<sup>2</sup>, where all the fruits could be easily monitored. Visits were recorded during series of 15 min of surveillance sessions at different hours of the day. A total number of 228 censuses, evenly distributed per population and year, were performed, corresponding to 57 h of net observation. During each session the following variables

were registered: total number of fruits under observation, number of visited fruits, ant species interacting with the seeds and ant's foraging behaviour. When individuals were detected removing and transporting the seeds, they were tracked and dispersal distances were measured. The ant species interacting with *P. vayredae* seeds were organized in functional groups from the plant point of view, according with their foraging behaviour and ability to transport and/or predate the seeds. In each group the interaction plant-ant was qualitatively classified as negative (complete removal of the elaiosome without seed transportation, or seed transportation to unsuitable places for germination), neutral (removal of diminutive portions of the elaiosome without seed transportation) or positive (effective seed transportation). Specimens were collected for further identification and vouchers were deposited at the Department of Biology, University of Aveiro.

#### *The effect of elaiosome removal in seed germination*

To analyse the effect of elaiosome removal in seed germination rates, after the fructification period of 2005, 10 germination plots containing both seeds with elaiosome and seeds without elaiosome (artificially removed) were randomly mounted along the population of Coldecarrera. Furthermore, seeds with the elaiosome removed by two ant species, *Crematogaster scutellaris* and *Formica gagates*, were sowed in two additional plots. These seeds were obtained during disperser's census, after seed manipulation by ants.

#### *Statistical analysis*

The primary dispersal distances among populations and years were analysed using Generalized Linear/Nonlinear Models (GLZ), with the dispersal distance adjusted to a Gamma distribution and a logarithmic link function for model responses. The Type 3 Likelihood Ratio test of effects was computed. Differences in dispersal distances and in the proportions of each dispersal unit (*i.e.*, capsule or seed) leaving the mother plant were analysed using a Wilcoxon rank-sum test and a z test, respectively.

The significance of the selective exclusion experiments, presence/absence of elaiosome, population and years on secondary seed removal was analysed using GLZ, with the dependent variable adjusted to a binomial distribution and a logit link function. The Type 3 Likelihood Ratio test of effects was computed. For each factor, when statistically significant differences were observed, the Kruskal-Wallis one-way ANOVA on ranks was performed to evaluate which groups were different. The Dunn's method was selected for post-hoc pairwise multiple comparisons.

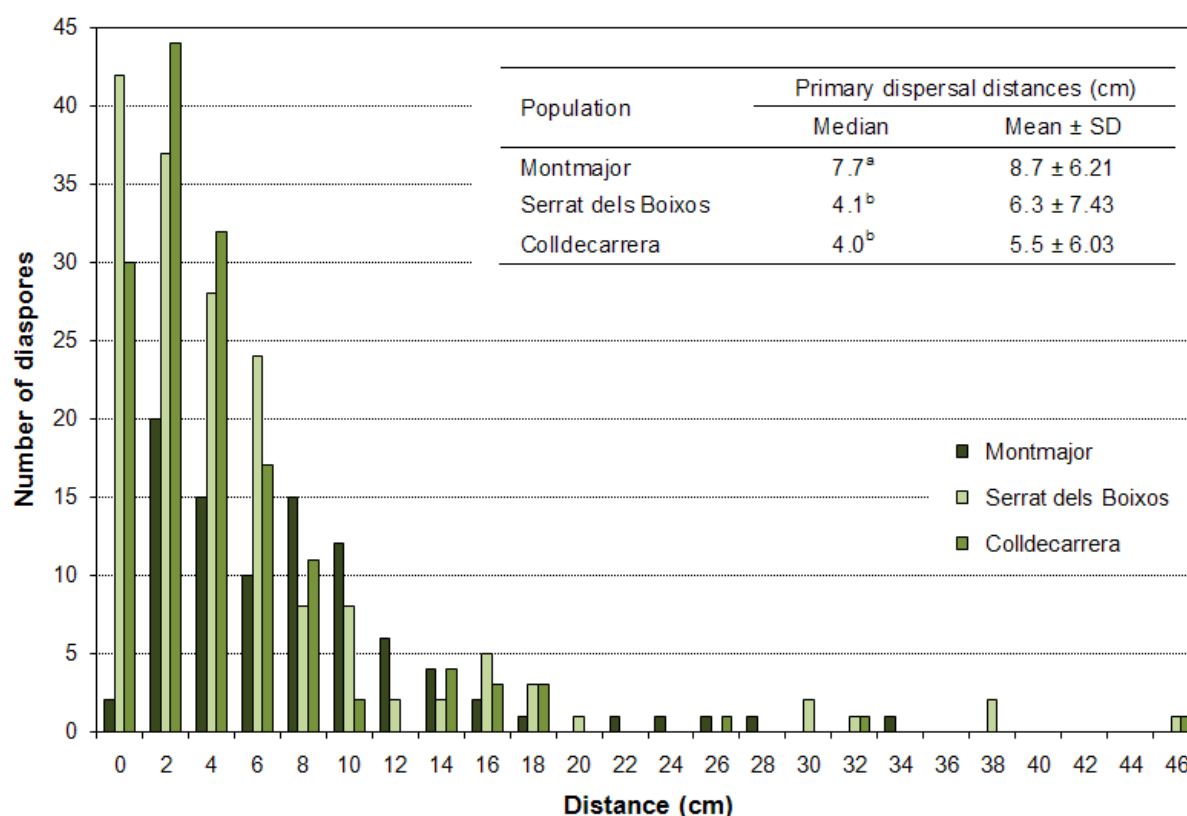
The frequency of ant-plant interactions (given as the number of visited fruits) among populations, years and ant's functional group were analysed using GLZ, with the dependent variable adjusted to a Poisson distribution and a logarithmic link function for model responses. The Type 3 Likelihood Ratio test of effects was computed. The dispersal distances observed in each functional group were analysed using a Kruskal-Wallis one-way ANOVA on ranks, followed by the Dunn's method for pairwise multiple comparisons.

Differences in germination rates among treatments (*i.e.*, seeds with intact elaiosome, elaiosome removed by ants and elaiosome artificially removed) were analysed with a one-way ANOVA. Prior to the analyses, germination rates were arcsine-transformed.

## Results

### *Primary dispersal*

During the process of maturation, the wings of the capsule start to open and expose the seeds. Overall, in the primary dispersal trays, 56.5% of the diaspores leaving the mother plant were the seeds, which fall mainly by gravity (barochory), while the remaining 43.5% of the diaspores were composed by the capsule containing at least one seed. These proportions were significantly different ( $z = 2.05$ ,  $P = 0.041$ ). Also, despite the capsule present wings, no statistically significant differences were observed in the dispersal distances between the two types of diaspores (seeds: mean  $\pm$  SD of  $5.9 \pm 6.69$  cm, median of 5.4 cm; capsule: mean  $\pm$  SD of  $6.7 \pm 3.00$  cm, median of 5.9 cm;  $T = 4,584.50$ ,  $P = 0.075$ ). The analysis of the variation of primary dispersal distances along time and space revealed statistically significant differences among populations ( $\chi^2 = 8.086$ ,  $P = 0.018$ ), but not among years ( $\chi^2 = 4.959$ ,  $P = 0.084$ ) nor in the interaction year  $\times$  population ( $\chi^2 = 5.810$ ,  $P = 0.214$ ). The primary dispersal curves obtained for each population were strongly skewed to the left (Fig. 5.1.1). Despite of the significant differences observed among populations, primary dispersal distances were limited in any population and resulted mainly from gravity: 95% of the diaspores fall within an 18 cm distance from the mother plant, and none of them were able to disperse to a distance greater than 46 cm (Fig. 5.1.1).



**Fig. 5.1.1** Histogram of primary dispersal distances of *Polygala vayredae* diaspores. An inset table, with the median, mean and standard deviation of the mean (SD) obtained for each of the studied populations, is also given. Medians followed by different letters are significantly different at  $P < 0.05$ .

#### Secondary dispersal and seed predation

The number of removed seeds from the selective exclusion treatments varied significantly among any of the studied factors (Table 5.1.1). In the studied populations, ants were found to be the main dispersers of *P. vayredae* seeds, with rodents being only punctually involved in seed removal/predation (Table 5.1.2;  $H = 211.3$ ,  $P < 0.001$ ). As expected, in the treatment that enabled free exposure of seeds to ants and rodents, the number of removed seeds was usually similar or slightly higher than what was observed in the treatment where ants were the only allowed group (Table 5.1.2). However, no statistically significant differences were observed between both treatments ( $P < 0.05$ ). It was also clear that the elaiosome played a major role in ant's attraction and subsequent seed transportation (76.2% of seed removal), as in its absence the number of removed seeds was significantly lower (42.6% of seed removal), independently of the population and year (Table 5.1.2;  $T = 67,774.5$ ,  $P < 0.001$ ). Spatial and temporal variations in the number of removed seeds were also observed. For instance, in 2005 the proportions of seed



removal were usually lower, especially in Serrat dels Boixos and Coldecarrera. Also, while ants were common dispersers and presented similar removal rates among populations, rodents were scarce, but more commonly involved in seed removal in Montmajor and Coldecarrera (Table 5.1.2).

**Table 5.1.1** Generalized Linear/Nonlinear Model performed on secondary seed removals for years, populations, selective exclusion treatments and presence/absence of elaiosome.

Effect	df	$\chi^2$	P
Year	2	111.22	0.0000
Population	2	43.41	0.0000
Treatment	2	1707.94	0.0000
Elaosome	1	421.44	0.0000





**Table 5.1.2** Secondary seed removals in the selective exclusion treatments investigated using seeds with or without elaiosome, in the studied populations during three consecutive years (2004-2006).

Elaosome	Exclusion treatment	Montmajor			Serrat dels Boixos			Coldecarrera			Sub-total
		2004	2005	2006	2004	2005	2006	2004	2005	2006	
Present	FE	8 ± 2.6	9 ± 3.0	6 ± 4.4	10 ± 0.5	6 ± 3.8	7 ± 3.4	9 ± 1.3	8 ± 2.5	8 ± 3.3	8 ± 3.1
	R	7 ± 3.8	8 ± 2.9	7 ± 4.2	8 ± 3.3	4 ± 4.2	10 ± 1.0	8 ± 2.9	6 ± 3.9	8 ± 3.1	7 ± 3.6
	A	2 ± 3.1	1 ± 3.3	0 ± 0.0	0 ± 1.0	0 ± 0.0	0 ± 0.0	3 ± 3.9	3 ± 4.1	0 ± 0.0	1 ± 2.5
	A+R	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Absent	FE	5 ± 4.4	5 ± 5.2	3 ± 3.2	3 ± 2.9	1 ± 1.7	6 ± 3.2	6 ± 3.5	3 ± 4.3	5 ± 4.1	4 ± 3.9
	R	5 ± 3.0	3 ± 3.4	6 ± 4.2	5 ± 3.5	0 ± 0.3	6 ± 3.9	3 ± 3.2	3 ± 3.6	7 ± 3.7	4 ± 3.8
	A	1 ± 2.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	1 ± 3.3	1 ± 3.0	0 ± 0.0	0 ± 1.6
	A+R	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0

Notes: Exclusion treatments: FE - free exposure to dispersers and predators, R - rodents exclusion, A - ants exclusion, C - ants and rodents exclusion. The number of removed seeds is given as mean and standard deviation of the mean over a value of 10 seeds offered in each treatment.

# Dispersal mechanisms in *Polygala vayredae*

**Table 5.1.3** Ant species, range of ant's body size (maximum and minimum), type and frequency of interaction (followed by the proportion of the total number of visits) and dispersal distances (mean  $\pm$  standard deviation of the mean, followed by the median) in each functional group observed in three populations during 2005 and 2006.

Species	Min-Max body size (mm)	Type of plant-ant interaction	Number of interactions						Dispersal distance (cm)	Photographs exemplifying each functional group
			Montmajor		Serrat dels Boixos		Colldecarrera			
			2005	2006	2005	2006	2005	2006		
<i>Crematogaster scutellaris</i> (Olivier)	2.9 - 5.2	-/0/+	1 (3.8%)	0 (0.0%)	9 (19.6%)	7 (14.3%)	46 (58.2%)	11 (19.0%)	62.5 ± 53.03 (62.5) <sup>a,b</sup>	
<i>Formica cunicularia</i> Latr.	4.0 - 7.5	++	1 (3.8%)	0 (0.0%)	15 (32.61%)	18 (36.7%)	17 (21.6%)	35 (60.3%)	415.9 ± 321.70 (459.0) <sup>a</sup>	
<i>Formica gagates</i> Latr.										
<i>Formica rufibarbis</i> Fabr.										
<i>Lasius alienus</i> (Förster)	2.1 – 5.0	+	20 (77.0%)	32 (82.0%)	12 (26.1%)	5 (10.2%)	8 (10.1%)	9 (15.5%)	57.6 ± 72.61 (24.5) <sup>b</sup>	
<i>Lasius emarginatus</i> (Olivier)										
<i>Lasius platythorax</i> Seifert										
<i>Myrmica spinosior</i> Santschi										
<i>Pheidole pallidula</i> (Nylander)	1.1 – 4.9	-/0	4 (15.4%)	7 (18.0%)	10 (21.7%)	19 (38.78%)	8 (10.1%)	3 (5.2%)	-	
<i>Plagiolepis pygmaea</i> (Latr.)										
<i>Tapinoma erraticum</i> (Latr.)										
<i>Temnothorax</i> spp.										
<i>Lasius flavus</i> (Fabr.)	1.7 - 14.0		No interactions were observed between these species and <i>P. vayredae</i> seeds							
<i>Aphaenogaster subterranea</i> (Latr.)										
<i>Camponotus aethiops</i> (Latr.)										
<i>Camponotus cruentatus</i> (Latr.)										
Total no. of analyzed fruits			122	169	268	140	231	313		
Total no. of interactions			26 (21.3%)	39 (23.1%)	46 (17.2%)	49 (35.0%)	79 (34.2%)	58 (18.5%)		

Notes: In each group the interaction plant-ant was qualitatively classified as negative (-), neutral (0) or positive (+), from the plant perspective for seed dispersal. The total number of observed fruits and interactions are also provided. Medians followed by different letters are significantly different at  $P < 0.05$ . Photographs exemplifying each functional group: (A) *Crematogaster scutellaris*, bar = 1 mm; (B) *Formica gagates*, bar = 1 mm; (C) *Myrmica* sp., bar = 1 mm; (D) *Temnothorax* sp., bar = 0.5 mm (photographs kindly provided by Crisanto Gómez).

*Disperser's assemblage, frequency of interaction and foraging behaviour*

A total number of 297 interactions were observed between the seeds of *P. vayredae* and ants during two consecutive years. A diverse array of ant species was observed visiting the fruits, each one enclosing specific foraging behaviours (Table 5.1.3) and responding positively to the presence of the elaiosome (*i.e.*, being attracted to the elaiosome). According with their foraging behaviour and dispersal ability, and from the plant perspective for seed dispersal, these species were arranged in four functional groups. The first group included the acrobat ant *Crematogaster scutellaris* (body size ranging from 2.9 to 5.2 mm), which presented several different actions (Table 5.1.3). In most cases, individuals of this species were observed removing parts or the complete elaiosome, leaving the seed inside the capsule, sometimes even before the capsule was completely mature (neutral to negative interaction); while in fewer occasions, they were seen actively transporting the seeds to the nest (negative interaction because the nests are constructed in tree trunks and, thus, the seeds are deposited in unfavourable sites for germination), eventually losing some of them in the way (positive interaction). The second group included several species from the genus *Formica* (Table 5.1.3), the larger ant species observed interacting with *P. vayredae* seeds (4.0 - 7.5 mm). These ants clearly recognized the seeds in the open capsules, actively transporting them through relatively large distances to their nests (positive interaction) (Fig. 5.1.2, Table 5.1.3). In the particular case of *Formica gagates* a curious behaviour was occasionally observed, with these ants accumulating the transported seeds in small pockets in the moss close to the nest. The third group included species from the genus *Lasius* and *Myrmica* (Table 5.1.3). These species behaved in a similar way as *Formica* spp., *i.e.*, they recognized and actively transported the seeds, but due to their smaller body size (2.1 - 5.0 mm), seeds were easily lost in the forest litter, resulting in much smaller dispersal distances (Table 5.1.3). The last group included the smallest ant species seen interacting with *P. vayredae* seeds (1.1 - 4.9 mm), which mainly belonged to the *Temnothorax* genus [*T. nylanderii* (Förster), *T. parvulus* (Schenck), *T. rabaudi* (Bondroit), *T. racovitzai* (Bondroit), *T. unifasciatus* (Latr.) cf.]. In this case, ants were unable to transport the seeds, but removed small parts of the elaiosome, progressively reducing its attractiveness (neutral to negative interaction) (Table 5.1.3). Another interesting feature of ant-seed interactions was the method of seed holding during diaspore transporting, which is determined by the relationship between the morphological feature and size of diaspores and by the size of the ant workers. While the larger ants (*Formica* group) grab the seed by the elaiosome, transporting them facing forward (see illustrations in Gorb and Gorb 2003), the smaller

ants have more difficulties to cope with the large size of the seeds, transporting them by reverse walking and dragging the seed along the substrate. The differences in dispersal distances among functional groups reported above were statistically significant ( $H = 31.69$ ,  $P \leq 0.001$ ), with the *Formica* group being able to transport the seeds to larger distances and the *Lasius* group to smaller ones (Table 5.1.3).

The number of interactions varied significantly between years ( $\chi^2 = 10.34$ ,  $P = 0.001$ ), populations ( $\chi^2 = 11.90$ ,  $P = 0.002$ ) and functional groups ( $\chi^2 = 11.73$ ,  $P = 0.008$ ). Despite of the temporal fluctuations observed in the number of interactions, from the spatial perspective, the most intense ant-plant interactions involved the *Lasius* and *Temnothorax* groups in Montmajor, the *Formica* and *Temnothorax* groups in Serrat dels Boixos, and the *Formica* and *C. scutellaris* groups in Coldecarrera (Table 5.1.3). Furthermore, in Montmajor and Serrat dels Boixos the number of interactions were higher in 2006, at least for the two major functional groups, while in Coldecarrera, the opposite was observed. In the latter population a shift in the main functional group was also observed (Table 5.1.3).



**Fig. 5.1.2** Dispersal of *Polygala vayredae* seeds by *Formica gagates*: (A) mature capsule with exposed seeds, (B) exploration of the seed by the ant, (C) seed detection, (D) seed removal, (E) seed transportation, and (F) empty capsule fixed to the mother plant.

#### *The effect of elaiosome removal in seed germination*

The germination rates in *P. vayredae* were highly variable (Table 5.1.4). No statistically significant differences were observed in the germination rates among elaiosome treatments (*i.e.*, not removed, artificially removed or removed by ants) (Table 5.1.4).

**Table 5.1.4** Germination rates of *Polygala vayredae* seeds with intact, artificially- and ant-removed elaiosomes.

Elaiosome manipulation	n	Seed germination (%)
Not removed	262	43.2 ± 21.15
Artificially removed	271	47.0 ± 28.47
Removed by ants	40	45.0 ± 30.00
One-way ANOVA		$F = 0.217, P = 0.807$

Notes: Seed germination is given as mean and standard deviation of the mean. The number of seeds sowed is also provided.

## Discussion

The study of the dispersal mechanisms operating in *Polygala vayredae* revealed new insights in the actual function of the morphological adaptations of its diaspores. In the absence of ants, the primary dispersal occurs mainly by barochory (*i.e.*, by the action of the diaspore weight), with the seeds being the main diaspores leaving the mother plant. This appears to result from the maturation process of the capsules, which opened the wings and exposed the mature seeds before leaving the mother plant. Furthermore, when the winged capsule was involved in primary dispersal, instead of the seeds, the dispersal distances did not increase in any of the studied populations. Therefore, it seems that, at present, the winged capsule is a non functional dispersal structure. Wind dispersal of the capsules prior to seeds release has been described as a dispersal mechanism in several species of *Polygala* and in other genera within the family (Sernander 1906; van Roosmalen 1985); nonetheless, experimental works confirming the actual functional importance of this syndrome are scarce. Wind dispersal of the capsules by means of the persistent wing-like sepals or by accidental epizoochory on the feet of grazing animals was observed in *P. vulgaris* (Sernander 1906; Lack and Kay 1987), and suggested to have important effects on the genetic structure of the populations (Lack and Kay 1987). Nonetheless, in those species wind dispersal has been described as an occasional occurrence, with myrmecochory being the principal mean of dispersal (Lack and Kay 1987; Paiva 1998). In *P. vayredae*, the significance of wind dispersal was largely limited by the growing strategy of the plant. The annual emission of shoots during the flowering period results in dense clusters of individuals, whose capsules are frequently found below the vegetative ramets or whose reproductive ramets are often bended on the soil. Furthermore, when certain ant species were exploiting for food and in the process

encountered the open capsules, they responded positively to the presence of the elaiosome and removed the seeds directly from the suspended capsules. Therefore, in *P. vayredae*, the seeds on the mother plant (primary dispersal) or in the soil (secondary dispersal) were actively searched and transported by several ant species (myrmecochory), which constitute the main dispersal vectors of this plant. It is worth noticing that the enclosure of the seed within the capsule could potentially maintain the attractiveness of the elaiosome for longer periods, especially in the exposed habitats, constituting a major advantage for myrmecochory. These observations can be interpreted as the consequence of evolutionary processes that lead to a loss in dispersal ability associated with habitat specialization.

Myrmecochory has been described as a diffuse mutualism present in over 3,000 plant species arranged in more than 80 families and distributed along a wide range of ecosystems (Berg 1975; Beattie 1985). In this mutualistic relationship, the elaiosome assumes particular importance in the attraction of the dispersal vectors, functioning both as the attractive part of the seed and as the reward offered for seed dispersal (Beattie 1985). Ant preferences for seeds with larger elaiosome/seed mass ratio (Mark and Olesen 1996) or the rejection of seeds without elaiosome (Hughes and Westoby 1992b; Wolff and Debussche 1999; Garrido *et al.* 2002) supports this hypothesis. This also implies that the traits involved in dispersal success, as elaiosome and seed size or the ratio between the two, will constitute the major targets of selection by ants (Edwards *et al.* 2006; Giladi 2006). In *P. vayredae*, the artificial removal of the elaiosome greatly reduced the rates of seed removal by ants (from 76% to 43%), revealing its importance as a successful trait for seed dispersal. Nonetheless, a significant proportion of seeds without elaiosome was also removed, indicating that different ant species presented distinct foraging behaviours. Similar patterns of seed removal were also observed in *P. vulgaris*, with the elaiosome playing a major role in ants attraction (86% of the seeds with elaiosome were removed), but with 56% of seeds without elaiosome being also transported (Oostermeijer 1989). Indeed, several studies have already shown that ant species may respond differently to the presence or absence of the elaiosome (*e.g.*, Hughes and Westoby 1992b; Garrido *et al.* 2002; Imbert 2006), revealing the need for future studies focused on assessing how the quantitative variation in seed traits affects seed removal and ants behaviour.

The assemblage and behaviour of the ant community interacting with the seeds of *P. vayredae* was found to be highly diverse. From the plant perspective for seed dispersal, it was possible to delineate four different functional groups, according with their ability to remove and transport seeds: two groups of effective dispersers, differing in their dispersal

distances, and two groups of elaiosome predators. As observed in other myrmecochorous species, the mean dispersal distances of *P. vayredae* seeds were rather limited (reviewed in Gómez and Espadaler 1998b). Nonetheless, some ant species were able to transport seeds to considerably large distances (up to 7.4 m). These observations are in accordance with the characteristic patterns of the dispersal curves produced by ants, *i.e.*, a curve positively skewed to short distances with a long tail (*e.g.*, Horvitz and Beattie 1980; Hughes and Westoby 1992a; Gómez and Espadaler 1998a). The dispersal distances attained by each ant species or group was mainly influenced by their body size (Ness *et al.* 2004) and by the method of seed holding during transportation (Gorb and Gorb 2003). Similarly to what was observed in *P. vayredae*, in *P. vulgaris*, Lack and Kay (1987) and Oostermeijer (1989) also reported a poor dispersal ability (up to 2 m), with *Lasius niger* (body size 3.4-5.0 mm; Domisthorpe 1927) being the main disperser in this case. Nonetheless, despite the differences in ant's body size and methods of diaspore transportation, the foraging behaviour, nest density, and/or spatial patterns of local ant communities may also be involved with the achieved dispersal distances (see Discussion in Gómez and Espadaler 1998a). Furthermore, to this complex equation of factors involved in plant-ant interactions and consequent dispersal capabilities, an additional feature should be contemplated, *i.e.*, elaiosome predation. In some cases elaiosome predation could result in a reduction of seed attractiveness, influencing further interactions with other ant species and ultimately affecting seed dispersal (S. Castro, personal observations and results herein). Overall, the limited dispersal ability observed in this species might be an important factor contributing to the formation/maintenance of its dense populations and to its restricted area of distribution, as observed in other narrow endemic species (*e.g.*, *Centaurea corymbosa*; Colas *et al.* 2000; Imbert 2006).

The assemblage of seed dispersers and the frequency of interactions between *P. vayredae* and each ant's functional group varied significantly at the spatial and temporal scales. Through the analysis of plant-ant interactions, one can assume that the ant community in each population was diverse, with the members of the *Lasius* group being the main effective dispersers in Montmajor, and the ones from the *Formica* group the most important in Serrat dels Boixos and Colldecarrera. Furthermore, in Colldecarrera a large number of interactions with *Crematogaster scutellaris*, a strong elaiosome predator, were also observed. These results are in accordance with previous studies that showed interpopulation variations in the composition of ant-dispersers assemblage (*e.g.*, Manzaneda *et al.* 2007). Spatial variation in the ant guilds may result from an array of different factors, namely habitat complexity (Lassau and Hochuli 2004), vegetation canopy

(Feener and Schupp 1998; Retana and Cerdá 2000), interspecific competition (Anderson 1992; Cerdá *et al.* 1998), temperature (Cerdá *et al.* 1998), and/or degree of anthropogenic disturbance (Andersen and Majer 2004). Despite an interaction between all these factors may be governing the spatial distribution of ant communities in the present study, it was evident that Montmajor population presents some specific characteristics that makes it dissimilar from Serrat dels Boixos and Colldecarrera. This population is found in a rocky slope, with small sized rupicolous vegetation, highly exposed to the sun, reaching high temperatures in some hours of the day. By other way, the remainder populations are found in shadowy meadows with *Pinus sylvestris*, and are maintained by human activity. Regarding the variation along time, it was mainly observed in Serrat dels Boixos and Colldecarrera, but, while in the former population it reflects general differences in the abundance of ants, in the latter it was also observed a shift in the leading group interacting with seeds of *P. vayredae*. Such differences have been already found in other myrmecochory systems and are usually associated with environmental parameters such as temperature, rainfall and/or moisture (Rico-Gray *et al.* 1998; Manzaneda *et al.* 2007). The observed spatial variation may lead to the selection of different dispersal traits among populations, as a result of different foraging behaviours within each population. On the other hand, the temporal variation in ant communities may potentially lead to shifts along time in the strength or direction of the selective pressures operating within each population. For example, recent research on the dispersal system of *Helleborus foetidus* in a macroecological scale has suggested that shifts in ants disperser assemblages are responsible for spatial differences in the selection of diaspore size (Garrido *et al.* 2002; Alcántara *et al.* 2007) and elaiosome chemistry (Boulay *et al.* 2006). Therefore, further studies on *P. vayredae* should focus on the evaluation of potential selective pressures operating in seed traits.

The role of seed predation and the impact of seed manipulation by ants in seed germination were also studied in the present work. Seed predation was a punctual feature during the studied period, and it did not lead to a significant loss of seeds. Thus, contrary to what has been observed in other species (*e.g.*, Heithaus 1981; Boyd 2001), predation of *P. vayredae* seeds by rodents is not among the selective pressures that are currently involved in ant mediated dispersal. Furthermore, granivorous ants are absent or extremely rare in the region inhabited by *P. vayredae* (X. Espadaler, personal observations). Regarding the influence of seed manipulation, no visible effects on the germination rates were observed in *P. vayredae*. While in several myrmecochorous plants, an enhancement in seed germination after elaiosome removal by ants has been described (*e.g.*, Culver and



Beattie 1980; Horvitz and Beattie 1980; Gómez *et al.* 2003), in others, negative and neutral effects have been recently reported (*e.g.*, *Centaurea corymbosa*, Imbert 2006; *Fremontodendron decumbens*, Boyd 2001). Overall, the relative contribution of each feature as selective advantages of myrmecochory varies with the plant species, the ant species and the ecosystem, with several advantages generally acting together in the evolution of this dispersal mechanism (Giladi 2006).

The distribution of plant species within populations and throughout their range of occurrence mainly depends on the distribution of suitable habitats for their establishment and growth, on their ability to disperse diaspores, and on their recent history processes (*e.g.*, van der Pijl 1982; Münzbergova 2004; Lesica *et al.* 2006). The present study provides valuable data on the dispersal mechanisms of the narrow endemic species *P. vayredae*. In the studied populations, this species did not seem to exhibit anemochory, prior to seeds release. Instead, either the seeds or the capsule fall beneath the mother plant by the action of their weight (barochory), or the seeds are directly collected in the suspended capsules by ants (myrmecochory). Thus, at the present, the winged capsule is not functional, and the plant is essentially myrmecochorous. A diverse array of ant species with different foraging behaviours was observed interacting with the seeds, with the elaiosome playing a major role in ant's attraction. Dispersal distances were limited and mainly determined by the ant's body size, but, contrary to the observed in the majority of myrmecochorous plants, considerable dispersal distances were achieved by some ant species. Spatio-temporal variations in the frequency of interactions were observed, which could eventually direct or disrupt the selection of seed traits. The obtained data provide the background information needed to further understand the distribution patterns, population structure and dynamics, gene flow and ability to reach new places on this species, ultimately providing important information for management strategies.

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## Chapter 6

### Concluding remarks



## Concluding remarks

The detailed study of the reproductive biology of *Polygala vayredae* revealed that this narrow endemic species presents remarkable features that determine its ability and success in sexual reproduction.

The flower revealed to be a very attractive and specialized structure. Its large dimension, strong and contrasting colours, large floral lifespan and nectar rewards, attract a diverse array of insects that could be potentially involved in its pollination.

Contrary to what has been described in several other species from the genus, *P. vayredae* is an obligatory xenogamic species, and thus, strictly relies on pollen vectors to achieve pollination. This results from an auto-incompatibility system that restrains the development of self pollen at the stigmatic papillae. This reproductive strategy presents a series of advantages and disadvantages. Despite lacking a reproductive strategy that enables the plant to produce seeds under unfavourable situations (*e.g.*, through delaying selfing or by the production of both chasmogamous and cleistogamous flowers), the production of seeds from unrelated gametes, only, will potentially maintain or improve the genetic diversity of the population and reduce the risk of inbreeding depression.

As a consequence of its reliance on animal vectors to attain pollination and of pollinator's scarcity, it was observed that *P. vayredae* is frequently subjected to pollen limitation, which detrimentally affects the fruit and seed production. Nonetheless, owing to its perennial habit, this species is able to overcome and persist through periods of pollinator unpredictability or scarcity. The analysis of *P. vayredae* flower longevity revealed that the plant appears to be specially adapted to this pollinator behaviour, as unpollinated flowers remain open for longer periods, increasing the opportunities to receive and export pollen. Despite of this, the reproductive costs of flower maintenance, as a result of delayed pollination, influenced the subsequent offspring.

Additionally, after pollination and during the progamic phase, the flowers of *P. vayredae* impose further constraints to seed production. After the self-incompatibility system blocks the development of self-pollen on the stigmatic papillae, the style physically restricts the access to a few pollen tubes only, potentially increasing the competition among male gametophytes at this level. At the ovary, strong interactions occur, determining the ovules that are fecundated. These features, despite limiting the offspring production, will probably be involved in the improvement of its quality.

In *P. vayredae*, the mechanism of secondary pollen presentation generally described as improving pollen delivery and receipt, revealed to bear its reproductive costs over both male and female fitness through intrinsic losses of gametes and self-interference. Adaptations, such as micro-herkogamy, could probably be involved in the improvement of the outputs of such a mechanism.

Regarding the floral visitors, the flowers of *P. vayredae* were visited by more than 24 different insect species. However, the present study revealed that this plant species frequently suffers from pollen limitation due to pollinator scarcity. This was mainly due to the inefficiency of most floral visitors when facing the closed corolla. The closed structure of the corolla form a long tube that must be triggered to achieve the nectar (or pollen), making this reward inaccessible to the majority of the observed floral visitors. Actually, only two species of long-tongued nectarivorous bees, *Bombus pascuorum* queens and *Anthophora* sp., and two species of pollinivorous, *Eucera longicornis* and *Halictus* sp., were able to move downwards the keel, get in contact with the reproductive organs and achieve pollination. The two groups of effective pollinators also revealed different behavioural features, which could lead to distinct impacts on the gene flow and on the reproductive outcome of the plant. The remaining floral visitors behaved as nectar larcenists (nectar robbers, secondary nectar robbers and nectar thieves) and were excluded from pollination, being however abundant in all the studied populations. Spatial and temporal variability in floral visitor's assemblage was observed, which lead to different reproductive outcomes among populations and years. Overall, the population located at higher altitude and at exposed rocky slopes was subjected to strongest pollen and pollinator's limitation, having an extremely low fruit and seed production.

Nectar rewards and consequently nectar robbers, revealed to be central features in the reproductive biology of *P. vayredae*. Nectar was an important trait in flower-visitor interactions, with legitimate pollinations being primarily influenced by this floral reward. Nectar robbing had important indirect and negative effects on plant fecundity, through both male and female functions. Negative correlations between robbing frequency and legitimate pollinations, and positive correlations between flower size and robbing frequency were observed. Thus, apparently, nectar robbers may be mediating an indirect negative selection over several phenotypic floral traits.

The analysis of dispersal mechanisms revealed that the diaspore syndromes are only morphological indicators of potential dispersal vectors and that their actual function must be evaluated to determine its real suitability. In the present study, it was observed that the



seeds or the capsules of *P. vayredae* fall beneath the mother plant by the action of their weight (barochory), or that the seeds are directly collected in the suspended capsules by ants (myrmecochory). Thus, presently, the functional adaptation of *P. vayredae* fruits to anemochory was largely reduced, leading to a limited capability of dispersion beyond the population limits. The plant is essentially myrmecochorous, with a diverse array of ant species being involved in seed dispersal, and with the elaiosome playing a major role in ant's attraction. Furthermore, considering the ability to disperse the seeds and the dispersal distances, several functional groups of ants were observed. From the plant's perspective, the majority of the ant species had a positive interaction with the seeds (e.g., *Formica* sp., *Lasius* sp.). The exceptions were *Crematogaster scutellaris*, which presented a diverse array of interactions and whose abundance varied along time and space, and the small *Themnothorax* species, which in general add an apparently neutral effect in further seed-ant interactions. Dispersal distances were generally limited and mainly determined by the ant body size. Spatio-temporal variation in the frequency of interactions was observed, which could eventually direct or disrupt the selection of seed traits.

The ability of *P. vayredae* plants to annually produce shoots from a rootstock linked with a dispersal mechanism that enhances seed dispersal within the population could be the main factors explaining the actual density of this species in the studied populations.

Further studies on seeds germination and survival, competition for pollinator with other flowering plants, as well as studies on the genetic structure of the main populations are being developed to increase the knowledge on this species.

The obtained data provides background information on the reproductive biology of the narrow endemic species *P. vayredae*. The reproductive strategy present in this endemic species largely restricts the production of fruits and seeds. Nonetheless, its several floral adaptations also improve the quality of the offspring that is produced. Thus, it seems that the plant "prefers" to produce fewer seeds, but with higher quality. On the other hand, its perennial habit allows it to overcome the periods of pollinator's scarcity, and thus persist over periods of low seed production. Furthermore, due to the short dispersal distances of the diaspores of *P. vayredae*, it seems that this species will be able to spread at rather slow rates beyond their actual distribution limits. Overall, future management programs should account with these issues, conserving the fauna and flora of the area and protecting plant-animal interactions. If so, the maintenance of the populations of *P. vayredae* can be sustained, and thus its conservation achieved.

## Future perspectives

Studies on the reproductive biology of endemic species are the first steps to understand its ecology and are essential to perceive potential problems in its life cycle. Nonetheless, they are part of a rather extensive investigation that should be developed in parallel to increase the knowledge on the biology of endemic, rare or threatened species. Demographic studies (Oostermeijer *et al.* 1996; Menges and Dolan 1998; García 2003) together with studies on the genetic structure of populations (Kang *et al.* 2005; Kim *et al.* 2005; Takahashi *et al.* 2005; Liu *et al.* 2006) have demonstrated to be very useful to understand the actual conservation status of threatened species and in the development of appropriate management strategies.

Furthermore, parallel studies integrating widespread congeners could also give insights on specific plant traits involved with rarity and risk of several species. Since the beginning of this century, some studies have already followed this approach and provided valuable information on explaining the causes of rarity on several plant groups (*e.g.*, Gitzendanner and Soltis 2000; Dodd and Helenurm 2002; Lavergne *et al.* 2003, 2004, 2005), with large scale projects being also currently in development (*e.g.*, Z. Münzbergova, *Prioritization of vascular plants for species conservation*).

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