

# Pollinators and crossability as reproductive isolation barriers in two sympatric oil-rewarding *Calceolaria* (Calceolariaceae) species

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**Abstract** Pollinator species are widely accepted as an important factor in plant reproductive isolation. Although mostly investigated in plants visited by different groups of pollinators (e.g., hummingbirds vs bees), few studies have examined the role of pollinators belonging to the same taxonomic group (e.g., only bees) on plant reproductive isolation. In this study, we investigate this question by evaluating pre- and post-zygotic mechanisms putatively involved in the reproductive isolation of two oil-rewarding sympatric *Calceolaria* species (i.e., *Calceolaria filicaulis* and *C. arachnoidea*) in an Andean ecosystem of Chile. We estimated reproductive isolation values using a combination of field (pollinator visitation rates) and experimental (intra and interspecific manual cross-pollination and seed germination of parents and hybrids) evidence. The two *Calceolaria* species were preferentially visited by different oil-collecting bee species. Results from hand cross-pollination experiments indicate that intraspecific crossings produced significantly more seeds than interspecific ones. Notwithstanding, seed germination essays did not reveal differences between parental and hybrids. Taken together, these results suggest that pollinator species are responsible for most of the reproductive isolation in the two *Calceolaria* species studied here. This study is the first assessment of pollinator-mediated reproductive

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isolation in *Calceolaria* species and the first to document reproductive barriers in oil-rewarding plants.

**Keywords** *Calceolaria* · Oil-collecting bees · Pollination · Reproductive isolation · Specialization

## Introduction

Reproductive isolation is a key component of the speciation process because it disrupts gene flow between otherwise interbreeding populations (Coyne and Orr 1998). Most cases of reproductive isolation in plants occur as a consequence of allopatric distributions. However, it has also been shown that different pollinators species can act as an effective isolation barrier in sympatry (Kay 2006; Ramsey et al. 2003), especially when pollinators exploit the same floral reward in different ways or at different times (e.g., Medel et al. 2003; Pauw 2006). Most studies addressing the evolution of reproductive isolation have been conducted in plants pollinated by divergent groups of pollinators, such as hummingbirds and bees in *Mimulus* (Ramsey et al. 2003) or hummingbirds and hawkmoths in *Ipomopsis* (Aldridge and Campbell 2006; but Scopece et al. 2007). Few studies have been performed on plants visited by pollinator species belonging to a genus or family (e.g., Costa et al. 2007; Sedeeq et al. 2014; Whitehead and Peakall 2014), although this is the ecological expectation in early diverging plant species. Such an example is the one of specialized pollination systems, in which reproductive isolation through mechanical and ethological divergence is observed, strengthening the interruption of gene flow between plant species. This situation was observed early by Grant (1949), Grant and Grant (1965) and Stebbins (1970), where changes in flower structure attracted different functional pollinator groups and promoted reproductive isolation through morphological adjustments between pollinators and flowers, as well as behavioral shifts in pollinators. Despite the growing interest on this topic, the role of pollinators as agents of reproductive isolation in plant species belonging to the same genus that exploit functionally similar pollinators has been rarely evaluated (see review in Lowry et al. 2011). Kay (2006) reported reproductive isolation in two hummingbird-pollinated and sympatric *Costus* species associated to small variation in floral morphology indicating that pollinators may be important agents of reproductive isolation even under slight floral divergence. Although rare, these studies are important as they illustrate the critical role that pollinator visitation frequency may have in promoting plant divergence or reinforcing selection after secondary contact.

*Calceolaria* is a diversified Andean genus whose nectarless flowers produce nonvolatile oils used as pollination reward only for solitary oil-collecting bees of the genera *Centris* and *Chalepogenus* (Rasmussen and Olessen 2000; Sérsic 2004). Although this interaction represents one of the most specialized plant-pollinator systems in the Americas (Cosacov et al. 2009), information on its ecology and evolution is scarce. Molau (1988), in an extensive taxonomic and morphological study, hypothesized that pollinators and habitat preferences are the main factors involved in the reproductive isolation of most *Calceolaria* species. Recent reports have shown that some *Calceolaria* species are indeed visited by different oil-collecting pollinators, which support an important element of Molau's hypothesis (Sérsic 2004; Murúa et al. 2014). However, although the role played by pollinators may be important in *Calceolaria*, the proximate mechanisms (e.g., pollinator

visitation frequency, post-zygotic unviability) responsible for reproductive isolation have never been examined in the group.

Here we use two largely allopatric, but locally sympatric *Calceolaria* species as model system to inquire into the role of pollinators as agents of reproductive isolation, in an attempt to identify one of the putative forces that molded the pattern of diversification of the genus in southern South America. Specifically, we evaluate reproductive barriers in the morphologically divergent *C. filicaulis* subsp. *filicaulis* Clos and *C. arachnoidea* Graham, and test the idea of pollinator-mediated reproductive isolation. Specifically, we ask whether or not pollinators are involved in the reproductive isolation of *C. filicaulis* and *C. arachnoidea* when in sympatry. To answer this question, (a) we examine the strength of different reproductive barriers in the field, (b) we perform intra- and interspecific pollination crosses in the greenhouse, and (c) we examine germination of parental and hybrid seeds as a measure of offspring viability.

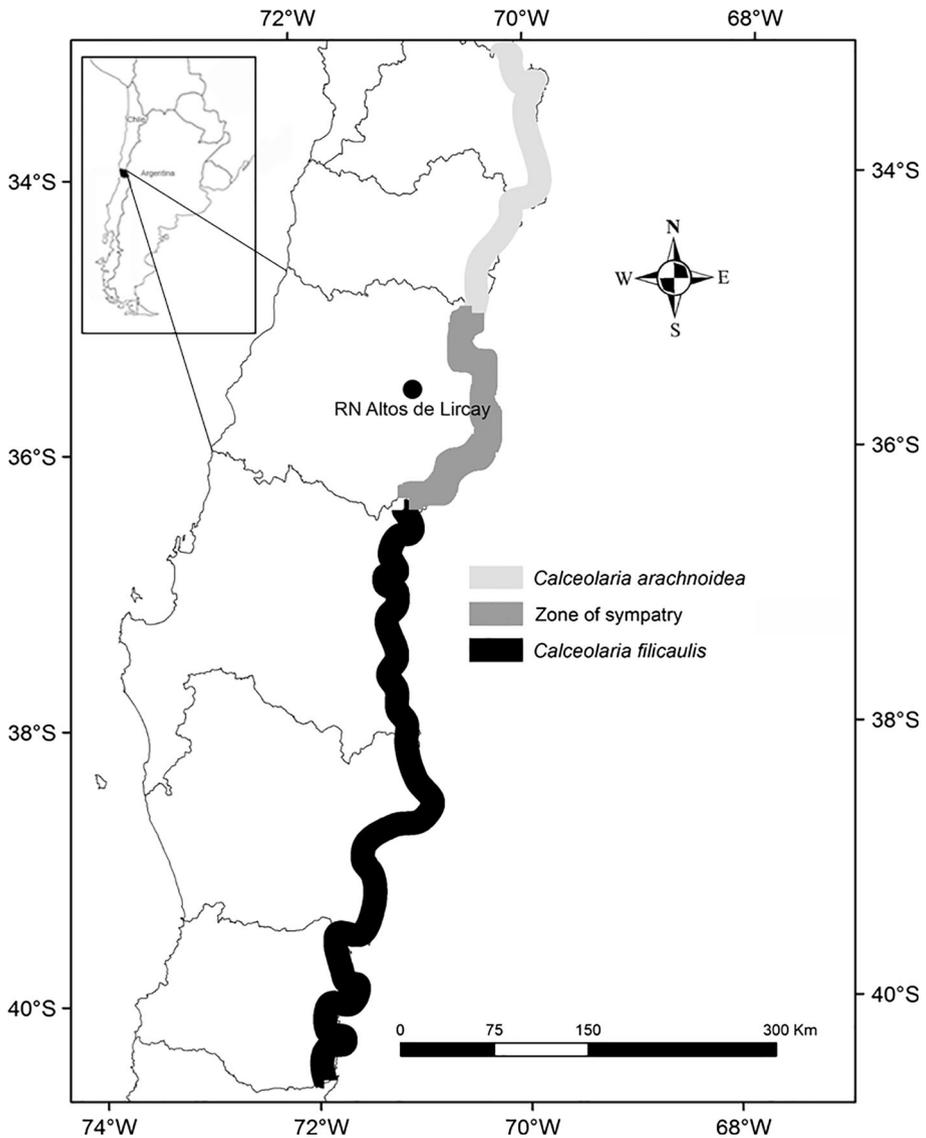
## Materials and methods

### Species and study site

*Calceolaria filicaulis* subsp. *filicaulis* (hereafter *C. filicaulis*) and *C. arachnoidea* are perennial herbs that grow close to streams and rivers in the Chilean Andes (30°–40°S). Except for some localities in central Chile, the two species have non-overlapping ranges (Ehrhart 2000; Fig. 1). Like all *Calceolaria* species, *C. filicaulis* and *C. arachnoidea* have zygomorphic flowers with a small superior lobe that covers the reproductive structures and an inflated lower lobe that contains the oil-secreting gland (Sérsic 2004). The two species differ in their type of mating system and several morphological traits, especially those related to the corolla and reproduction (Fig. 2). *C. filicaulis* is a self-incompatible and strictly outcrossing species with a small yellow corolla, with lobes spaced apart and the oil gland enclosed in the lower lobe and located close to the corolla aperture, which facilitates oil acquisition by pollinators (Murúa et al. 2014; Fig. 3a, b). *C. arachnoidea* is a self-compatible species with a mixed mating system that displays a large purple corolla, with lobes tightly closed and the oil gland situated at the end of the lower lobe in a position hardly accessible to pollinators (Fig. 3c, d).

This study was conducted during the spring-summer season of 2012 at Altos de Lircay National Reserve (35°36'S, 71°00'W, 2200 m., Figure 1), in Chile. In the study locality, *C. filicaulis* and *C. arachnoidea* are sympatric and form discrete patches, surrounded by a vegetation dominated by *Mimulus luteus* and *M. cupreus* (Phrymaceae), *Hypochoeris acaulis* (Asteraceae), *Pozoa coriacea* (Apiaceae) and *Azorella incisa* (Apiaceae).

The putative hybrids between *C. filicaulis* and *C. arachnoidea* display flowers with a reduced yellow upper lobe and an inflated red lower lobe (Fig. 3 e–f). Even though hybrids have been described as relatively frequent in nature (Ehrhart 2000), they were scarce in the study site (14 plants with and without flowers) compared to the more abundant *C. filicaulis* (254 plants) and *C. arachnoidea* (88 plants), and showed low reproductive output (mean  $\pm$  SE; 52  $\pm$  11 seeds per plant) in comparison to their putative parental species (mean  $\pm$  SE; *C. filicaulis*: 290.4  $\pm$  34.07 seeds per plant; *C. arachnoidea*: 500.82  $\pm$  54.68 seeds per plant).

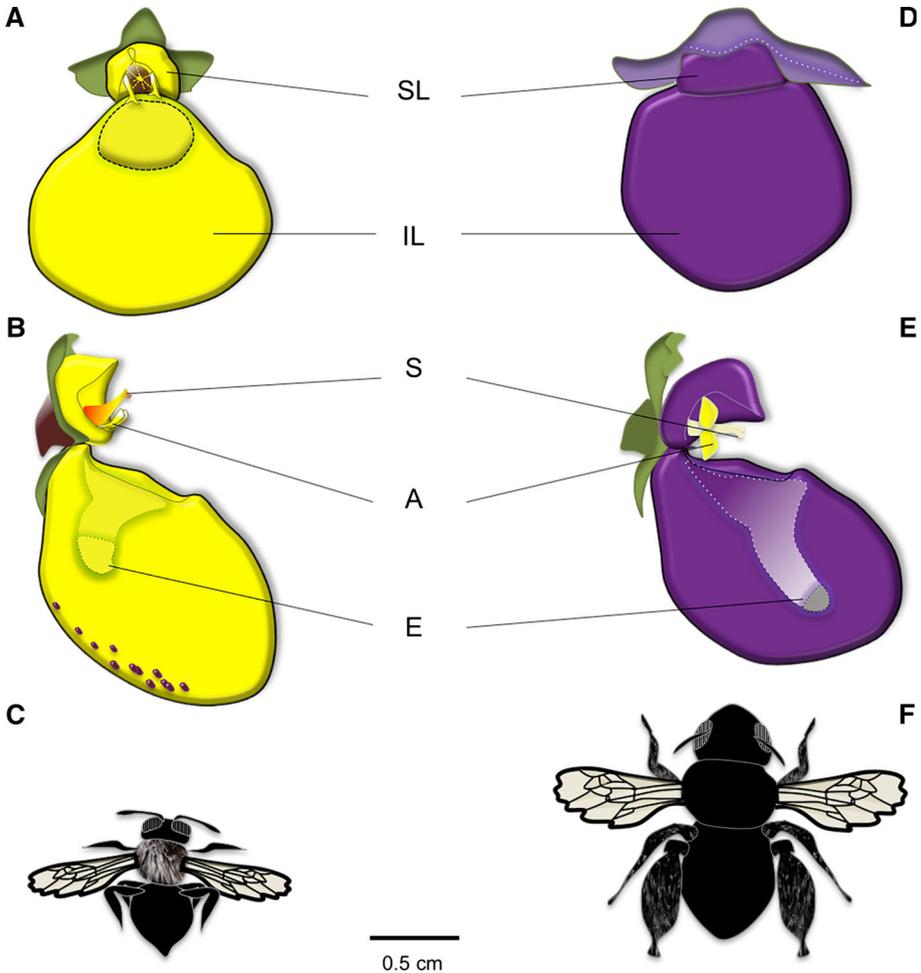


**Fig. 1** Geographical distribution of the two studied *Calceolaria* species and their sympatric zone Ehrhart (2000)

## Reproductive isolation barriers

### *Pre-mating isolation: floral visitors*

We recorded all floral visitors in both *Calceolaria* species during January 2012. Focal observations of 15 min per plant were performed during six sunny days from 09:00 to 18:00 h for a total of 50 h of observation per species. Only insects that opened the two



**Fig. 2** Frontal (a, d) and lateral (b, e) views of corolla parts and the two principal oil-collecting bees (c, f) of each study *Calceolaria* species. a–b *Calceolaria filicaulis*, d–e *Calceolaria arachnoidea*, c *Chalhepogenus caeruleus*, and f *Centris nigerrima*. Superior (SL) and Inferior lobe (IL), as well as the position of style (S), anthers (A) and elaiophore (E) are indicated

floral lobes and entered the flower were recorded. Samples of pollinator species were collected and taken to the laboratory for taxonomic identification.

To quantify the reproductive isolation due to differences in the identity of floral visitors we used a pollinator isolation index ( $RI_{pollinator}$ ) following Kay (2006), which ranges from 0 (no isolation) to 1 (complete isolation), as follows:

$$RI_{pollinator} = 1 - (\text{proportion of visits made by a shared pollinator species})$$

Additionally, in order to determine if any pollinator that visit both *Calceolaria* species were also visiting the few flowering hybrids found in the field (five plants), we performed focal observations during five consecutive days (10 h of observation). Unfortunately, this



**Fig. 3** Habitus (a, c, e) and detail of floral morphology (b, d, f) of the two studied *Calceolaria* species and the putative hybrids found at the study site. a–b *C. filicaulis*, c–d *C. arachnoidea*, and e–f hybrids

preliminary pollinator survey led to no observation of any insect visiting the putative hybrids.

#### *Post-mating isolation: seed production*

We assessed the level of post-mating isolation through a cross-pollination experiment. We grew plants of both species in the greenhouse using seeds collected in the field from 40 capsules, each from different individuals. Twenty-five seeds per capsule were randomly chosen and sown in 40 seeding pots ( $40 \times 25 = 1000$  seeds sown per species). Seeds were germinated under daily watering and constant temperature at 18 °C. After 3 weeks, seedlings were transplanted into individual growing pots until flowering (% germinated

seeds per species: *C. filicaulis* = 30.96%; *C. arachnoidea* = 28.17%). Then, 60 plants of each species were randomly chosen and assigned to one of the following treatments: (1) intraspecific crossing between different plants of *C. filicaulis* (n = 30 plants), (2) intraspecific crossing between different plants of *C. arachnoidea* (n = 30 plants), (3) interspecific crossing with *C. filicaulis* as pollen donor (n = 15 plants) and *C. arachnoidea* as pollen receiver (n = 15 plants) and (4) interspecific crossing with *C. arachnoidea* as pollen donor (n = 15 plants) and *C. filicaulis* as pollen receiver (n = 15 plants). For all treatments, three buds of the receiver plant were emasculated and pollinated with pollen obtained from three flowers of the donor plant. Ripe fruits were harvested and their seed production counted. The effect of parental source on the mean seed production of each pollination treatment was tested using a Generalized Linear Model (GLM) with Poisson distribution and log function in R version 2.15, glm function in package stats version 3.4.0 (R Development Core Team 2011). Even though this design does not permit the separation of treatment and maternal effects, the low number of flowers per plant produced by *C. filicaulis* (mean =  $1.9 \pm 0.3$  flowers/plant) precluded us from setting a balanced design using three replicates per treatment per plant. Finally, the reproductive post-mating isolation index ( $RI_{postpollination}$ ) following Kay (2006) was estimated as:

$$RI_{postpollination} = 1 - \left( \frac{\text{\#seeds by interspecific pollination}}{\text{\#seeds by intraspecific pollination}} \right)$$

where  $RI = 0$  indicates no isolation and  $RI = 1$  indicates complete postpollination isolation.

#### *Post-mating isolation: seed germination*

We carried out germination experiments in the greenhouse to quantify potential reproductive isolation attributable to low germination success of hybrids. In doing so, we used a sample of the seeds produced by both intra and inter-specific crosses obtained by the cross-pollination experiment described above (see “[Post-mating isolation: seed production](#)” section). Since a preliminary essay showed that seedling emergence was about the same in pots having 30 or 10 seeds, we standardized estimates to 10 seeds per capsule. Thus, 10 seeds per capsule coming from every pollination treatment (i.e., intra and inter-specific) performed in the post-mating isolation: seed production essay (60 plants per species: 15 plants per treatment; see above) were sown and grown in individual seeding pots under constant temperature (18 °C). After the radicle emerges (12 days approximately since sowing), the number of seedlings produced by hybrids (coming from inter-specific crosses) and parental (coming from intra-specific crosses) plants were recorded and their proportion per treatment quantified. The effect of the parental source on the proportion of successful seedlings belonging to intra- and interspecific pollination treatments was analyzed using a GLM with Binomial distribution and logit link-function in R. Finally, we calculated a  $RI_{viability}$  isolation index as the proportion seedlings produced in each treatment (Kay 2006), as follows:

$$RI_{viability} = 1 - \left( \frac{\text{F1 hybrid seedlings}}{\text{Parental seedlings}} \right)$$

where  $RI = 0$  indicates complete hybrid viability (no isolation) and  $RI = 1$  indicates complete isolation.

Confidence intervals for all *RI* indexes (i.e., visitation, seed production, and proportion of successful seedlings) were estimated using 10,000 bootstrap replications in R (script available as supplementary material).

## Results

### Pre-mating isolation: floral visitors

All flower visitors were hymenopterans, four of them belonging to the Apidae family and one to the Megachilidae family (Table 1). *Calceolaria filicaulis* was visited by only one species (*Chalepogenus caeruleus*). *Calceolaria arachnoidea* was visited by three bee species (*Centris nigerrima*, *Megachile semirufa* and *Chalepogenus herbsti*), the principal pollinator being *Centris nigerrima* (99% of visits). Only three (*Centris* and *Chalepogenus*) out of four floral visitors in both species were oil-gathering pollinators. The bumblebee *Bombus terrestris* was an occasional visitor in the two *Calceolaria* species. However, the large body size of this species turns unfeasible its participation in the pollen transference between plants. In consequence, *RI*-values were estimated in the absence of *B. terrestris*. The estimation of  $RI_{pollinator}$  showed the same value ( $RI = 1$ ) for both *Calceolaria* species (Table 2).

### Post-mating isolation: seed production

Both *Calceolaria* species produced fewer seeds in the interspecific than in the intraspecific crossings under greenhouse conditions. The significant Donor x Receiver interaction term

**Table 1** Floral visitors of *Calceolaria filicaulis* and *C. arachnoidea*

Plant species	Pollinators	Family/tribe	Visitation rate [visits/ (flower <sup>-1</sup> h <sup>-1</sup> )]	Proportion of visits
<i>C. filicaulis</i>	<i>Chalepogenus caeruleus</i>	Apidae/ Tapinotaspidini	0.29 ± 0.07	1.00
<i>C. arachnoidea</i>	<i>Centris nigerrima</i>	Apidae/Centribini	0.30 ± 0.05	0.991
	<i>Megachile semirufa</i>	Megachilidae/ Megachilini	0.0005 ± 0.0005	0.002
	<i>Chalepogenus herbsti</i>	Apidae/ Tapinotaspidini	0.002 ± 0.002	0.007

The identity of pollinators, taxonomical classification, mean visitation rate (±SE) and the proportion of visits are shown

**Table 2** Indexes of reproductive isolation for the two sympatric *Calceolaria* species

Isolating barriers	Strength of reproductive isolation ( <i>RI</i> )	
	<i>C. filicaulis</i>	<i>C. arachnoidea</i>
Pollinator	1.00	1.00
Seed production	0.74 (0.53–0.90)	0.91 (0.86–0.95)
Seed germination	0.11 (–0.62–0.66)	0.16 (–0.71–0.59)

Reproductive isolation indexes, *RI*; 0, absence of reproductive isolation, and 1, complete reproductive isolation. Parentheses indicate confidence intervals

**Table 3** Summary of GLM effects of parental source (D: donor, R: receiver) and their interaction (D × R) on the seed production and germination of *Calceolaria* species

Seed production (N = 30)	Estimate	$\lambda$ value	P
D	-5.16	-66.36	<0.001
R	-6.67	-59.72	<0.001
D*R	3.81	61.95	<0.001
Germination (N = 30)	Estimate	z value	P
D	-0.32	-0.16	0.87
R	-1.03	-0.53	0.60
D*R	0.24	0.19	0.85

N indicates the number of plants used in each treatment (see “Materials and methods” section)

in the GLM revealed that the level of reproductive isolation was dependent on which species acts as pollen donor or pollen receiver (Table 3). Intraspecific crossings on *C. filicaulis* showed a seed production almost four-fold higher than that obtained from interspecific crossings (mean  $\pm$  SE;  $453.62 \pm 52.11$  seeds/fruit and  $117.75 \pm 40.98$  seeds/fruit, respectively). Likewise, seed production from intraspecific crossings in *C. arachnoidea* was 11-fold higher than interspecific crossings ( $303.35 \pm 50.87$  seeds/fruits and  $25.97 \pm 5.10$  seeds/fruit, respectively). Thus the contribution of seed set to the  $RI_{postpollination}$  barrier differed between plant species, being higher in *C. arachnoidea* (0.91) than in *C. filicaulis* (0.74) (Table 2).

### Post-mating isolation: seed germination

Germination success did not differ between treatments (Table 3). Seeds from intraspecific crosses in *C. filicaulis* germinated in a similar proportion to those coming from interspecific crosses with *C. filicaulis* as the pollen receiver (mean  $\pm$  SE;  $0.39 \pm 0.06$  seeds/fruit and  $0.35 \pm 0.12$  seeds/fruit, respectively, Table 3). Likewise, the proportion of germinated seedlings from intraspecific crossings in *C. arachnoidea* ( $0.27 \pm 0.07$  seeds/fruit) was similar to that from interspecific pollination ( $0.23 \pm 0.05$  seeds/fruit) (Table 3). Finally,  $RI_{viability}$  values were low for both *Calceolaria* species: 0.11 for *C. filicaulis* and 0.16 for *C. arachnoidea* (Table 2), indicating that germination plays a minor role in the reproductive isolation between species.

## Discussion

In this study, we investigated whether functionally similar pollinators can represent successful reproductive isolation barriers in two plant species belonging to the same genus. To do this, we tested the relative role of pollinators in the reproductive isolation of two locally sympatric plant species of genus *Calceolaria*. Our results demonstrate that although post-mating isolation is present between species, visits by different pollinators play a central role in interspecific isolation. Our results agree with the few studies done that indicate that pollinators can be important in allowing divergence and maintaining isolation in specialized pollination systems. Below, we discuss our results in further detail.

## Pre-mating isolation: floral visitors

The two *Calceolaria* species were visited by different oil-collecting bee species. Specifically, in the field, we did not observe cross-visitations in spite of the fact that plants of both species locate near to each other without microhabitat segregation. This is reflected in the maximal  $RI_{pollinator}$  values ( $RI = 1$ ) recorded for each species. Excepting for studies performed in orchids (see Scopece et al. 2007), these RI-values are high in comparison to most studies assessing the role of pollinators as agents of reproductive isolation. In our study, the high RI-values may be explained by the high dependence of the *Calceolaria* species for a circumscribed group of oil-collecting bees. Recent investigations have suggested that the floral morphology of *Calceolaria* species is what restricts the access of oil-collecting bees to floral resources (Murúa and Espíndola 2015), causing the observed high specificity in this plant-pollinator interaction. From a more general perspective, our results are consistent with studies performed in more generalist plant-pollinator systems such as *Ipomopsis* (Aldridge and Campbell 2006), *Mimulus* (Ramsey et al. 2003), *Narcissus* (Marqués et al. 2007), *Pedicularis* (Yang et al. 2007), *Penstemon* (Chari and Wilson 2001). Along with this, our study also agrees with the conclusions reached by Lowry et al. (2011), which in a review of 19 cases of isolation by pollinators conclude that the composition of floral visitors is an important reproductive barrier in most cases.

Both *Calceolaria* species were mostly visited by one single bee species (Table 1). At least two reasons may explain the differences observed between species. First, species appear to display different volatile cues, such as the sweet smell produced by *C. filicaulis* only (Murúa, pers. obs.), which could be playing a role of attraction for *Chalepogenus* but not for the other bee species. Even though we do not have quantitative information to support this observation, it is likely that specific volatiles promote ethological isolation in *Calceolaria* as reported in other pollination systems (e.g., Schiestl and Schlüter 2009, Byers et al. 2014; Bischoff et al. 2015). Second, the floral architecture of *C. filicaulis* seems to favor *Chalepogenus* pollination as it provides a better landing platform than the more globular corolla of *C. arachnoidea*. Indeed, it has been reported that *Chalepogenus* tend to associate with *Calceolaria* flowers having a protruding, flat and wide lower lip (Sérsic 2004; Murúa and Espíndola 2015), similar to the one present in *C. filicaulis*. On the other side, even though *Centris* is a large-sized bee that can easily manipulate globular and flat flowers, it is often observed on closed and globular *Calceolaria* species, such as *C. arachnoidea*. Flowers of *C. arachnoidea* are grasped with the middle legs and flower lobes are open due to insect weight, which permits the free access to the elaiophore (Murúa, personal observations). In addition, despite pollination effectiveness was not estimated, the small size of *Chalepogenus* suggests a higher effectiveness on *C. filicaulis* as *C. arachnoidea* requires pollinators with large body size and weight to open their tightly closed flower lobes.

Interestingly, the two *Calceolaria* species were occasionally visited by the buff-tailed bumblebee *Bombus terrestris*, a generalist non-oil-collecting exotic pollinator that was introduced to Chile in the 1990s (Montalva et al. 2011; Esterio et al. 2013). *Bombus terrestris* is a large-sized bumblebee that has spread quickly over the Chilean territory (Schmid-Hempel et al. 2014). In recently colonized habitats, *B. terrestris* shows a conspicuous variation in abundance across years, probably as consequence of local biotic and abiotic constraints that prevent the full establishment in the first steps of the invasion process (Esterio et al. 2013). Although we have not evaluated the abundance nor pollination effectiveness of this bumblebee, its presence in the study site suggests that, in

principle, it may disrupt the isolation barrier created by *Centris* and *Chalepogenus* species. However, the strong pre-mating isolation barriers reported in this study together with the inherent specialized association between *Calceolaria* and oil-collecting bees (Molau 1988; Sársic 2004; Murúa and Espíndola 2015) turns unfeasible this possibility.

In spite of the strong pollinator isolation observed between the two *Calceolaria* species, we cannot rule out that changes in the RI values occur across years and/or localities. Since our study was restricted to 1 year only and one locality, interpretation of our results should be performed with caution. Currently, there is ample agreement that pollinator abundance and species composition often change in response to subtle variations in the biotic and abiotic environment (e.g., Herrera 1988; Petanidou et al. 2008; Olesen et al. 2008). In Chile, much of the inter-annual variation is largely influenced by El Niño events (Escobar and Aceituno 1988), which is characterized by cold and rainfall seasons followed by dry and hot years. Under this scenario, it is likely that pollinator species composition varies according to climate fluctuation, allowing the arrival of new species such as other oil-collecting bees not recorded in the study site. Likewise, spatial variation in the composition of pollinator assemblages and plant communities may modify the pollen dynamics between the two *Calceolaria* species, resulting in different hybridization patterns and isolation barriers. Studies that include more localities across consecutive years are needed to examine the consistency of our estimates.

### Post-mating isolation: seed production

We found post-mating isolation in both directions, although the successful seed production appeared to depend on the pollen receiver (higher with *C. filicaulis* as pollen receiver; Table 3). These results indicate that although pollinators may play an important role in maintaining the two *Calceolaria* species reproductively isolated, other mechanisms may also participate in the process. We observed relatively high RI seed production values in both species, with higher values for *C. arachnoidea* (0.91) than *C. filicaulis* (0.74). Our results are similar to those estimated by Kay (2006) for *Costus pulverulentus* (0.95) and *C. scaber* (0.87), Martin and Willis (2007) for *Mimulus nasutus* (0.94) and *M. guttatus* (0.96), and by Scopece et al. (2013) for *Orchis maculata* (0.8), but higher than those obtained by Ramsey et al. (2003) for *Mimulus lewesii* (0.41) and *M. cardinalis* (0.49).

Interspecific crossings produced fewer seeds than intraspecific ones. Two general physiological-morphological mechanisms have been suggested to explain this pattern (Arnold 1997). First, interspecific differences in the ability of pollen tubes to reach the ovary seem to result from the fact that species with long styles will produce pollen tubes adapted to grow longer than species with short styles. In consequence, long-styled species can easily fertilize plants with short styles, but not the converse (e.g., Howard 1999; Chari and Wilson 2001; see Tiffin, Olson and Moyle 2001). In our study, the style length of *C. filicaulis* is shorter than that of *C. arachnoidea* (mean  $\pm$  SE; *C. filicaulis*:  $1.62 \pm 0.04$  cm; *C. arachnoidea*:  $2.43 \pm 0.05$  cm), which is consistent with variation in seed production between species in cross-pollination experiments. Second, pollen competition has been suggested as a strong post-mating barrier (e.g., Campbell et al. 2003; Ramsey et al. 2003). When conspecific and heterospecific pollen compete in the same style, heterospecific pollen tubes may grow at a slower rate or show greater attrition than conspecific ones because of a lack of adaptation to the stylar tissue (Chari and Wilson 2001). Examples of pollen competition have been widely documented in different species such as *Chamerion* (Husband et al. 2002), *Louisiana* (Emms et al. 1996) and *Mimulus* (Diaz and Macnair

1999). The extent to which this mechanism is present in the species studied here needs to be assessed in future studies.

### Post-mating isolation: seed germination

Our results revealed that the proportion of germinated seedlings of hybrids did not differ from that of parental plants. Judging by the low isolation indexes shown by *C. filicaulis* (0.11) and *C. arachnoidea* (0.16), hybrid germination contributed to a small extent to reproductive isolation between the species. These results contrast with those observed in *Costus* (Kay 2006), *Orchis* (Scopece et al. 2013), *Pitcairnia* (Wendt et al. 2001) and *Antirrhinum* (Carrió and Güemes 2014), where hybrids had lower germination success than parental plants (but see Burke et al. 1998). One possible explanation for the discrepancy of our results with those of previous studies may relate to differences in the time of divergence between species. As the strength of post-zygotic barriers depends to a large extent on the time of divergence and hence on the genetic distance between taxa (reviewed in Widmer et al. 2009), it is likely that the separation time of the two *Calceolaria* species is still too recent to preclude hybrid germination and viability. Unfortunately, the evolutionary relatedness of most *Calceolaria* species is not fully understood, which precludes genetic contrasts in this group.

In this study we reported on the role of pollinators as agents of reproductive isolation in specialized pollination systems, using two sympatric *Calceolaria* species as model system. With a combination of observational and manipulative approaches, we documented that *Calceolaria* species are mostly isolated by their principal floral visitors, reinforcing the idea that pollinator identity plays a major role in the reproductive isolation of plant species. While the evolutionary consequences of the levels of reproductive isolation detected in this study are clearly beyond the focus of this work, it is likely that divergent pollinator preference shown by *Chalepogenus* and *Centris* species conveys reinforcement selection on *C. filicaulis* and *C. arachnoidea* through premating isolation barriers.

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