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**EVOLUCIÓN DE LAS INTERACCIONES PLANTA-
POLINIZADOR EN UN SISTEMA ESPECIALIZADO:
EL CASO DE *CALCEOLARIA* EN CHILE.**

Tesis

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A handwritten signature in black ink, consisting of a large, stylized 'R' followed by several vertical lines, positioned above a horizontal line.

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A complex, multi-layered handwritten signature in black ink, positioned above a horizontal line.

*“Soy,
las ganas de vivir
las ganas de cruzar
las ganas de conocer
lo que hay después del mar...”*

(R. Pérez Joglar)



*a todos quienes trabajaron, colaboraron y apoyaron
cada parte de este trabajo*



Nací un 23 de Julio de 1981 en Antofagasta, ciudad donde realicé mis estudios de pregrado para el 2006 titularme como Ecóloga Marina en la Universidad de Antofagasta. El 2008 me incorporé al laboratorio de Ecología Evolutiva del Dr. Rodrigo Medel e ingresé al Magister en Ciencias Biológicas de la Universidad de

Chile. Durante los primeros años su laboratorio me entregó la oportunidad de trabajar en diferentes proyectos de investigación, participar en la realización de manuscritos y actividades de extensión, los cuales dieron inicio a mi desarrollo científico. Finalmente, el año 2009 y bajo la tutoría del Dr. Medel, decidí ingresar al Doctorado en Ciencias de la Universidad de Chile, donde continué mi formación académica con miras a ampliar mis conocimientos y encaminar lo que a futuro se convertiría en mi línea de investigación. Actualmente, mis intereses y esfuerzos están dirigidos a estudiar los procesos evolutivos que subyacen a las interacciones planta-polinizador, principalmente en sistemas especialistas, a modo de comprender la manera en que estas interacciones evolucionan, se adaptan y mantienen en el tiempo.

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RESUMEN

Los polinizadores han sido considerados como los principales agentes responsables de la evolución y diversificación floral. De un punto de vista teórico, la evolución floral puede ser libre o estar restringida por la historia filogenética de las especies en cuestión. Por otro lado, es sabido que el proceso de especiación es el resultado de la acción conjunta de diferentes barreras de aislamiento reproductivo, tanto pre- como post-cigóticas. De esta forma, el aislamiento entre especies aparece como el producto de diferentes barreras que pueden variar en magnitud e importancia, dependiendo de la especie en cuestión.

En general, los mecanismos de evolución floral y su relación con los polinizadores han sido principalmente estudiados en sistemas de polinización generalistas. Por cierto, muy pocos estudios han sido realizados en sistemas especializados. Teniendo en cuenta lo aquí mencionado, el objetivo general de esta tesis es comprender la evolución floral en un sistema planta-polinizador especialista, evaluando la hipótesis que la misma haya ocurrido en respuesta a sus polinizadores. Para esto, se usó como modelo de estudio la interacción de polinización presente entre varias especies chilenas del género *Calceolaria* y sus abejas recolectoras de aceites. En este estudio se intentó entonces i) determinar los factores responsables de la diversificación floral en *Calceolaria* y examinar las relaciones ecológicas que mantienen la especificidad de la interacción y, ii) establecer posibles mecanismos de especiación para las especies del género *Calceolaria*.

Para responder al objetivo i) se reconstruyeron las relaciones filogenéticas de nueve especies de *Calceolaria* y estimó la fuerza de su señal filogenética. Adicionalmente, se cuantificaron las relaciones estructurales entre la morfología floral

y la de sus polinizadores. El objetivo ii) fue estudiado con la cuantificación de algunas barreras de aislamiento reproductivo (tres pre-cigóticas y una post-cigótica). Nuestros resultados indican ausencia de señal filogenética, lo que sugiere que la relación ancestro-descendiente juega un papel menor en la evolución de los rasgos morfológicos en el sistema de estudio. Desafortunadamente, no encontramos correlación entre los caracteres florales y la morfología de los polinizadores, lo cual nos impide concluir que los polinizadores son la principal fuerza evolutiva actuando sobre la evolución de caracteres florales en *Calceolaria*. No obstante, esto último no puede ser descartado ya que la relación entre *Calceolaria* y sus polinizadores ha sido descrita como una de las más recientes asociaciones entre plantas secretoras y abejas recolectoras de aceites, por lo que dicha relación podrían estar aún en pleno proceso de ajuste. Finalmente, el análisis de barreras de aislamiento reproductivo mostró que las especies se encuentran principalmente aisladas por barreras pre-cigóticas, lo que indicaría que los polinizadores juegan un rol clave en la mantención de la identidad de las especies en simpatria.

Globalmente, estos resultados sugieren que la evolución y diversificación floral del género *Calceolaria* puede haber ocurrido principalmente en respuesta a contingencias ecológicas, más que a relaciones ancestro-descendencia, quizás en relación con sus principales polinizadores. Esta idea es sugerida por el rol importante que los polinizadores juegan como barrera interespecíficas de aislamiento reproductivo (capítulo IV). No obstante, el rol último de los polinizadores como agentes de selección sobre el fenotipo floral en especies del género *Calceolaria* es aún un tema en estudio.

ABSTRACT

Pollinators have been considered as the main factors in the evolution and floral diversification. However, this change may have morphological restrictions due to the phylogenetic history shared by the species. For its part, the morphological differences between species can be understood under the concept of pollination syndromes. This concept has been widely studied for different groups of plants. However, its applicability in species pollinated exclusively by a single functional group of pollinators has received little attention. In this sense, it is known that the speciation process is the result of the joint action of different reproductive isolation barriers, both pre- and post-zygotic. Then, the insulation between species appears as the product of different barriers that can vary in magnitude and importance depending on the species.

In general, the mechanisms of floral evolution and its relationship to pollinators have been mainly studied in generalist pollination systems, and very few studies have been conducted in specialized systems. Based on the above, in this thesis, the overall goal is to understand the floral evolution in a specialized plant-pollinator system and test the hypothesis that such evolution has occurred in response to their pollinators. For this, we used the pollination interaction present between the plant genus *Calceolaria* and its oil-collecting bees as a study model. In this study, we thus attempted to i) determine the factors responsible of floral diversification and examine the ecological relationships which maintain the high specificity of the interaction and, ii) determine possible mechanisms of speciation in the genus *Calceolaria*.

To investigate objective i) we constructed the phylogenetic relationships of nine species of Chilean *Calceolaria* and we estimated their phylogenetic signal. In

addition, we quantified the structural correlation between the plant and pollinator morphology. The objective ii) was study by quantifying the magnitude of some reproductive isolation barriers (three pre-zygotic and one post-zygotic) between two focal *Calceolaria* species. Our results indicate the absence of phylogenetic signal, suggesting that the ancestor-descendant relationship plays a minor role in the evolution of morphological traits. Unfortunately, we did not found correlation between plant and pollinator traits, reason why is not possible to conclude if pollinators are the main evolutionary force acting in *Calceolaria* trait evolution. However, the latest could not rule out due to the relationship between *Calceolaria* and their pollinators has been described as one of the most recent relationship between oil-flowering and oil-collecting bees, therefore this relationship would be still in a matching process. Finally, the analysis of reproductive isolation barriers showed that the studied species are mostly isolated by pre-zygotic barriers, where pollinators play a major role in maintaining the identity of sympatric species.

Overall, these results suggest that the evolution and floral diversification of the genus *Calceolaria* may have occurred in response to ecological contingency, more than to ancestral-descendant relationship, may be in relation to their principal pollinators. This idea was strongly supported by the observation of the major role played by pollinators in the reproductive isolation of species (Chapter III). However, the ultimate role of pollinators as selective agent of floral phenotype in species of the *Calceolaria* genus is still a subject of study.

CAPITULO I

INTRODUCCION

El estudio de las interacciones planta-polinizador ha sido de gran interés en biología evolutiva debido a la gran diversidad que éstas presentan, a su rol en la evolución de las plantas con flores, así como también su influencia en los patrones de flujo genético entre las especies (Kay & Schemke 2003). Desde una perspectiva general, las relaciones planta-polinizador pueden clasificarse como generalistas (involucrando muchos grupos de plantas y de polinizadores) o especialistas (involucrando sólo un grupo reducido de organismos; Johnson & Steiner 2000). Las interacciones generalistas han sido ampliamente estudiadas, debido a que la gran mayoría de las especies presentan este nivel de interacción. Mientras que, las interacciones especialistas son raras en la naturaleza, por lo que son menos estudiadas. Esto resulta particularmente desafortunado dado que los sistemas especialistas representan excelentes modelos para estudiar la forma a través de la cual las interacciones inter-específicas evolucionan, se adaptan y se mantienen en el tiempo (Pellmyr et al. 1996).

Históricamente, se ha propuesto que la diversidad de fenotipos florales habría evolucionado como una adaptación a la polinización animal, y que tal asociación sería un factor clave en la diversificación y radiación de las Angiospermas (Darwin 1862, Stebbins 1970, Dood et al. 1999). Diferentes líneas de evidencia provenientes de diversos estudios micro y macroevolutivos (ver revisión en Johnson 2010) han confirmado el rol de los polinizadores como agentes de cambio evolutivo. Sin embargo, también se ha propuesto

que la evolución de los rasgos florales podría estar afectada a restricciones filogenéticas entre las especies (Fenster et al. 2004). A pesar de que esta última teoría fue propuesta hace ya varios años, escasos son los estudios que han evaluado la influencia de restricciones filogenéticas en la evolución de caracteres relevantes para el proceso de polinización (e.g., Larson & Barret 2000, Steiner et al. 2011). En este contexto, el estudio de los caracteres individuales puede proveer buena información para comprender la evolución de la morfología floral como un todo. Más aun, determinar la magnitud, labilidad y los patrones de correlación entre caracteres florales, así como también la de estos con la morfología de sus polinizadores, puede sin duda propiciar al mejor entendimiento de los procesos que han conducido la diversificación floral y especiación de las plantas con flor (Pérez et al. 2006, Alcantara & Lohmann 2010).

La relación entre adaptación y especiación continúa siendo un tema central en biología evolutiva. Se sabe que el proceso de especiación implica mucho más que una diferenciación fenotípica como resultado de la adaptación. Por esta razón, en las últimas décadas el estudio del proceso de especiación se ha focalizado en determinar el rol y la fuerza de diferentes barreras de aislamiento, las cuales definirían o mantendrían los límites de las especies (Johnson 2010). Relacionado con esto, se ha demostrado que el potencial flujo génico entre poblaciones inter-fértiles puede cesar por la presencia de mecanismos de aislamiento pre- y post-cigóticos, los que actuarían como barreras reproductivas en cualquier estado del ciclo de vida de las plantas (Dobzhansky 1937). El aislamiento entre especies aparece entonces como el producto de diferentes barreras pre- y post-cigóticas, que pueden variar en magnitud e importancia dependiendo de las especies (Coyne & Orr 1998). Esto significa que determinadas barreras son capaces de reducir -pero no

necesariamente impedir- el flujo génico entre especies, lo cual indica que para determinar su rol en el proceso de especiación es necesario cuantificar la magnitud e importancia relativa de las múltiples barreras de aislamiento (Widmer et al. 2009). Esto adquiere relevancia cuando se estudian especies filogenéticamente emparentadas que habitan en simpatria, donde barreras como aislamiento geográfico, ensamble de polinizadores, aislamiento mecánico mediado por diferencias en la morfología floral, entre otras, tienen un rol fundamental en la mantención de la identidad de las especies involucradas.

Los mecanismos de evolución floral y su correlación con los polinizadores han sido principalmente estudiados en sistemas de polinización generalistas. Sin embargo, muy pocos estudios han abordado estos temas en sistemas de polinización especializados (ver Armbruster et al. 2009), lo que resulta desafortunado, ya que estos representan excelentes modelos biológicos para comprender los factores que favorecen el establecimiento y mantenimiento de interacciones inter-específicas entre plantas y polinizadores.

Uno de los ejemplos de sistema altamente especializados es aquel formado por la interacción entre plantas secretoras de aceites y sus polinizadores (Renner & Schaefer 2010). El desarrollo de glándulas secretoras de aceites o elaioforo, se encuentra en más de 1500-1800 especies de plantas pertenecientes a 11 familias (Renner & Schaefer 2010, Chauveau et al. 2011). Mientras que, la recolección y utilización de aceite es realizado por abejas solitarias pertenecientes sólo a dos familias: Melittiidae y Apidae (Michener 2007). Por lo tanto, estos sistemas consisten en plantas que ofrecen aceites florales como recompensa a la polinización, en vez de néctar, siendo visitadas casi exclusivamente por abejas colectoras de aceites, quienes utilizan la recompensa para la alimentación de sus larvas y la construcción de nidos (Vogel 1969). En general, estas abejas presentan

estructuras especiales en forma de peines o cerdas, ubicadas en el bitarso de las extremidades delanteras y medias, las que actúan como recolectores de aceites (Vogel 1974). La mayoría de estas abejas generalmente utilizan el primer y segundo par de patas para realizar la recolección (e.g., Centridini), a excepción de Ctenoplectidae y Tapinotaspidini, cuyas especies utilizan el conjunto de cerdas presentes solo en las patas delanteras (Vogel 1990, Machado 2004). Respecto a la especificidad de la interacción, se ha observado que esta varía de acuerdo a la especie de polinizador, dado que muchas de estas abejas recolectan aceites en un amplio ensamble de plantas (Machado 2004). No obstante, en casos como el de las especies de *Diascia* y su polinizador *Rediviva pallidula*, se ha observado una fuerte correlación entre el largo de patas y la distancia a la posición del elaioforo, sugiriendo que la distancia a la recompensa podría determinar la evolución del largo de las patas en esta especie, en un contexto coevolutivo (Steiner & Whitehead 1990, Steiner & Whitehead 1991).

Otro ejemplo de interacción especializado se encuentra representado por el género de plantas *Calceolaria* (Calceolariaceae) y sus abejas polinizadoras. *Calceolaria* es un grupo altamente diversificado en el continente americano y se compone de aproximadamente 250 especies, que se encuentran distribuidas desde México hasta Tierra del Fuego. La mayoría de las especies de este género están relacionadas con hábitats andinos asociados a cursos de aguas como arroyos y ríos. Pero también se les encuentra en zonas costeras del Pacífico y en la costa atlántica del sur de Argentina (Sérsic 2004). A pesar de que estas especies presentan un amplio rango de distribución, muchas de ellas habitan en simpatria, donde es posible observar en algunos casos la presencia de fenotipos intermedios (Ehrhart 2000). El número de relaciones filogenéticas y sus relaciones de

parentesco han sido recientemente descritas por Cosacov et al. (2009) quienes combinando caracteres morfológicos y moleculares advierten la presencia de tres subgéneros: *Calceolaria*, *Cheiloncos* y *Rosula*, los que agrupan especies de diversas formas, tamaños y colores. En general, las flores del género *Calceolaria* presentan una corola zigomorfa bilabiada, compuesta por un pequeño lóbulo superior y un gran lóbulo inferior inflamado, el cual contiene el elaioforo (Vogel 1974). Las especies difieren en muchos de sus rasgos morfológicos, especialmente aquellos relacionados con el tamaño de la corola, posición del elaioforo y posición de las estructuras reproductivas, que se relacionan a diferentes estrategias reproductivas que van desde la autoincompatibilidad completa hasta la alogamia parcial (Sérsic et al. 2001, M. Murúa, datos no publicados).

La gran mayoría de las especies de *Calceolaria* son polinizadas exclusivamente por abejas solitarias recolectoras de aceites del género *Centris* y *Chalepogenus* (Himenóptera: Apidae; Rasmussen & Olesen 2000, Sérsic 2004), las cuales varían en el tamaño y posición de las estructuras morfológicas responsables de coleccionar los aceites, así como también en sus conductas de forrajeo (Rasmussen 1999). Por ejemplo, *Centris* presenta peines compuestos por pequeñas cerdas, situados ya sea en el primer par de patas o en las patas delanteras y medias, dependiendo de la especie (Sérsic 2004). El recolector de aceite se encuentra situado en la superficie ventral del bitarso y consiste en dos peines, uno en la parte posterior y otro en la anterior. Mientras que, *Chalepogenus* presenta colectores de aceites solo en las patas delanteras, el que consiste en un conjunto de cerdas generalmente situadas en el bitarso, a excepción de algunas especies donde las cerdas se extienden hacia el meditarso y la tibia (Coccuci et al. 2000). En cuanto a la conducta de forrajeo, *Centris* sigue rutas fijas de vuelo cubriendo extensas áreas donde realiza visitas cortas a la mayoría

de las flores presentes en una inflorescencia. Una vez que aterriza en las flores, *Centris* utiliza las patas del medio para sujetar la corola e inserta las patas delanteras para recolectar el aceite, el cual traspasa hacia las patas traseras durante el vuelo (Sérsic 2004). Por el contrario, *Chalepogenus* no presenta rutas de vuelo fijas y limita sus visitas a pequeñas poblaciones de plantas, visitando todas las flores que puede dentro de los parches. Debido a su pequeño tamaño aterriza con poca precisión sobre las flores y no es capaz de sostener la corola con sus patas. La recolección de aceites la realiza desde el borde de la apertura de la corola, la cual es interrumpida por intervalos de transferencia de aceite desde las patas delanteras a las traseras sin abandonar las flores (Sérsic 2004).

En general, *Calceolaria* y sus polinizadores representan particular interés por tres razones. De un punto de vista biogeográfico, se han reconocido dos centros de diversificación: uno más antiguo en las latitudes medias de Chile, y otro más reciente en el norte de Perú (Molau 1988, Cosacov et al., 2009). Segundo, la posibilidad de que el género haya diversificado a partir de la interacción con abejas recolectoras de aceites. Esto último, basado en la observación de que ciertas *Calceolarias* que habitan la zona norte de la distribución han perdido la glándula probablemente en relación a la ausencia o escases de abejas colectoras de aceites (Molau 1988), lo cual sumado a que es sabido que la presencia de tricomas es una condición ancestral en este género permite sugerir que estas especies podrían haber evolucionado a partir de la presencia de abejas recolectoras de estos aceites (Cosacov et al. 2009). Tercero, estudios recientes han constatado que los aceites florales son colectados sólo por unas pocas especies de abejas solitarias de los géneros *Centris* y *Chalepogenus*, quienes serían entonces los principales responsables de la polinización de estas especies (Sérsic 2004).

En Chile, existen aproximadamente 50 especies con 20 taxa intraespecíficos (Ehrhart 2000), distribuidas desde la costa hasta la cordillera de los Andes entre 26° y 51° de latitud. En esta distribución el género representa un excelente lugar para el estudio de la evolución de la interacción entre las especies de *Calceolaria* y sus polinizadores por las siguientes razones. Primero, el linaje más antiguo del género corresponde a especies chilenas, desde donde al parecer colonizaron y diversificaron al resto de América (Cosacov et al. 2009). Segundo, en esta zona existe una gran diversificación de especies con un alto porcentaje de endemismo (*i.e.*, 74% de las especies descritas están presentes solo en Chile; Molau 1988, Ehrhart 2000). Paralelamente, existe una gran diversidad de formas, colores, tamaños y posición de la glándula secretora de aceite, lo que en su conjunto hacen de las *Calceolarias* chilenas, un modelo de estudio adecuado para poner a prueba la hipótesis de delimitación de especies (Ehrhart 2000). Finalmente, las especies chilenas son polinizadas por una pocas especies de abejas de los géneros *Centris* y *Chalepogenus*, cuya presencia y abundancia están espacialmente diferenciadas (Sérsic 2004).

Basado en lo anterior, los objetivos principalmente de esta tesis son: determinar los factores responsables de la diversificación floral, examinar las relaciones ecológicas que sostienen la alta especificidad entre plantas y polinizadores y, establecer posibles mecanismos de especiación para las especies del género *Calceolaria*. Para ello, este trabajo se divide en dos capítulos que abarcan los objetivos anteriormente mencionados.

El primer capítulo examina los factores proximales responsables de la evolución de caracteres florales en especies del género *Calceolaria*, con el objetivo de evaluar si la variación morfológica observada en este grupo está principalmente explicada por la historia filogenética entre las especies o por sus polinizadores. Para ello se reconstruyeron las

relaciones filogenéticas de nueve especies chilenas pertenecientes al subgénero *Rosula*. Posteriormente, se determinó el efecto de la filogenia en la evolución de los rasgos florales de las especies estudiadas. Finalmente, para examinar las relaciones ecológicas que sostienen la especificidad entre plantas y polinizadores, se cuantificó la estructura de correlación parcial entre los caracteres florales y la de sus principales polinizadores.

El segundo capítulo intenta explorar los factores responsables de la mantención de las especies en sistemas de polinización especialistas. Así, con el objetivo de determinar la importancia relativa de cada una de ellas en la prevención del flujo génico inter-específico en situación de simpatria, se utilizaron como modelos dos especies de *Calceolaria* altoandinas (*C. arachnoidea* y *C. filicaulis*), que han sido observadas en simpatria en la región chilena del Maule. En este sistema se cuantificó la magnitud de cuatro barreras de aislamiento reproductivo: tres pre-cigóticas (aislamiento por microhábitat, polinizadores y polinización intra e interespecíficas) y una post-cigótica (viabilidad de la F₁).

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CAPITULO II

PATTERNS OF FLORAL EVOLUTION IN OIL-REWARDING *CALCEOLARIA* SPECIES

ABSTRACT

Pollinators have been invoked as the principal factor affecting floral evolution. However, floral trait diversification may be constrained by phylogeny. *Calceolaria* is a diverse American genus whose flowers are pollinated by a specialized group of oil-collecting bees. To determine the proximal factors driving the evolution of floral traits in this system, we examined i) the extent to which the floral morphological variation is explained by their history or by ecological contingency and ii) how floral and insect morphology relate each other. For this, we first reconstructed the phylogenetic relationships of these plant species based on nuclear and chloroplastic DNA sequences. In addition, we tested the hypothesis of phylogenetic signal of each floral trait. Afterwards, we evaluated the correlation between some floral traits of nine *Calceolaria* species and different traits from their principal pollinators. Our results indicate an absence of phylogenetic signal, suggesting a minor role of ancestor-descendant relationship and a major role of ecological contingency in the evolution of morphological traits. Unfortunately, we did not find correlation between plant and pollinator traits, reason why is not possible to determine if pollinators are the main evolutionary force acting in *Calceolaria* trait evolution. Despite of that, these findings

suggest that *Calceolaria* floral diversification might have evolved primarily in response to its ecological context, but the role of its specialized pollinators is still a subject of study.

KEYWORDS: Phylogenetic reconstruction, *Calceolaria*, oil-collecting bees, phylogenetic signal, specialization.

INTRODUCTION

Plant reproductive success in exogamous species is strongly related to the potential of flowers to attract and manipulate floral visitors (Faegri & van der Pijl 1966, Stebbins 1970, Fenster et al. 2004, Kay & Sargent 2009). Different lines of evidence have confirmed that pollinators represent an important force driving the evolution of floral traits (see review in Harder & Johnson 2009). Recent studies on pollinator-mediated selection have demonstrated that pollinator preferences for specific phenotypes induce significant covariation between characters and plant fitness in floral traits, such as floral display (Reynolds et al. 2010, Nattero et al. 2011), corolla color (Campbell et al. 1997), floral shape (Gómez et al. 2006, Murúa et al. 2010), corolla size (Armbruster et al. 2005, Nattero et al. 2010), nectar guide (Medel et al. 2003), among others. Likewise, studies at the macroevolutionary level have reconstructed the putative influence of pollinators on floral evolution (see Trip & Manos 2008, and references therein), suggesting that pollination shifts seem to be involved in floral trait diversification (Armbruster et al. 2009, Márten-Rodríguez et al. 2010).

In spite of the fact that pollinators are one of the most important proximal factors affecting floral evolution (van der Niet & Johnson 2012), floral trait diversification may be constrained by phylogeny (Fenster et al. 2004). For example, Steiner et al. (2011) suggested that the phylogeny strongly explained the variation of floral scents in oil-secreting orchids. Although the hypothesis of plant-pollinator co-diversification could not be rejected in that system, it appeared that the evolution of floral scents had a strong phylogenetic signal, despite the high specialization of the plant-pollinator system. Larson & Barret (2000)

examined the extent to which phylogeny influenced pollen limitation in 224 species belonging to diverse families. Their results indicate that history weakly explained pollen limitation albeit a significant variation in the intensity of pollen limitation was detected in seven families, suggesting that some phylogenetic component might be involved in the process.

The study of floral trait evolution in a phylogenetic context has been mainly conducted under the framework of pollination syndromes (e.g., Pérez et al. 2006, Marten-Rodriguez et al. 2010). This practice has provided important insights into the evolution of floral specialization. However, the relationship between a single functional pollinator group with closely related plant taxa has been less investigated (but see Armbruster et al. 2002). This is unfortunate as comparative methods at this level may be helpful to understand the way specialized interactions arise and the factors involved in such process (Smith 2010).

This study focuses on a specialized plant-pollinator system between the oil-rewarding plant genus *Calceolaria* L. and the oil-collecting bees of genera *Centris* and *Chalepogenus* (Hymenoptera: Apidae; Rasmussen & Olesen 2000, Sérsic 2004). *Calceolaria* is a large American genus, which presents different life forms (shrubs, subshrubs and herbs; annual or perennial) and displays flowers of a wide color and shape diversity (Molau 1988, Sérsic 2004). In general, flowers are bilabiate with an inflated lower lip, which bears an oil-secreting gland, the elaiophore (Vogel 1974). Recent phylogenetic relationships inferred by Cosacov et al. (2009) found support for three subgenera, *Calceolaria*, *Cheiloncos* and *Rosula*, the last presenting a high variability in color, shape and corolla size. Variation in floral shape and color, together with the visit of unusual pollinators make species of *Rosula* an appropriate model to explore the potential role of pollinators in the evolution of floral

traits.

The aim of this study is to determine the proximal factors driving the evolution of floral traits in a specialized plant-pollinator system. For this, we examine the extent to which the high morphological variation observed in this plant group is explained by the history of the group or by ecological contingency. To do so, we reconstruct the phylogenetic relationships of nine *Calceolaria* species of the subgenus *Rosula* and test for phylogenetic signal of different floral traits on the same nine species. If any phylogenetic signal was detected we explored the evolutionary mode of this floral traits, if not, the ecological relationships between floral traits and pollinator morphology was explored through partial correlation analysis.

MATERIALS AND METHODS

Sampling

The study was conducted during the spring-summer seasons of 2011-2013 and included nine *Calceolaria* species and two subspecies (one population per species/subspecies) present in Central and Southern Chile (Table 1). Species and subspecies selection based upon the phylogenetic relationships reported by Cosacov et al. (2009). In each population, ten and twenty plants were randomly chosen for leaf material collection and digital floral trait measurement based on pictures, respectively. Following the work of Cosacov et al. (2009), *Jovellana punctata* was used as outgroup for phylogenetic analysis.

Morphological traits

Five floral and four pollinator traits were measured from pictures using the software ImageJ 1.46r (available in: <http://rsb.info.nih.gov/ij/>; Figure 1 and 2). The floral traits were: corolla area (CA) measured from a frontal perspective, herkogamy (distance between reproductive structures; H), elaiophore area (area; EA), distance between the stigma and the elaiophore (DSE), and distance between the anthers and the elaiophore (DAE).

In each population, and during five consecutive sunny days, we recorded and captured all the insects observed visiting and touching the reproductive structures of each *Calceolaria* species. All pollinators were preserved in ethanol 70% and later identified to the lowest taxonomic level. We identified and classified pollinator species as either oil- or pollen-collecting bees. Based on pictures taken under a binocular microscope, we measured: total length (TL), thorax width (TW), maximum extension for oil collection (MOC), and length of the oil-collecting zone (LOZ; Figure 2). Specifically, the maximum extension for oil collection (MOC) was estimated as the total length of the leg plus the thorax height (TH). Because the two groups of oil-collecting pollinators have different behavioral and morphological adaptations, the length of the oil-collecting zone was considered as an indicator of the amount of oil they are able to collect. Indeed, and as mentioned above, while *Centris* displays oil-collecting combs in the first and the second leg pairs, *Chalepogenus* presents the oil-collecting structures only in the first leg pair (Sérsic 2004). Based on this, the length of the oil-collecting zone (LOZ) was calculated from the sum of the values for the first two leg pairs in *Centris* and for the first one only in *Chalepogenus*. Because this feature is absent of pollen-collectors, we did not measure LOZ in such cases.

DNA extraction, amplification and sequencing

Leaf material was collected and dried in silica gel, and DNA extractions were done following a modified protocol by ATG Genetics Inc ©. Two nuclear regions (nDNA) and four chloroplastic regions (cDNA) were amplified using published standard primers (Sup. Mat. I): the entire ITS and 5.8s regions (White et al., 1990), a fragment of *matK* (Andersson 2006), *rpl32f-trnL*, *rpl32r-ndhF* and the intergenic spacer *trnS-G* (Shaw et al. 2007; Sup. Mat. I). DNA samples were amplified in a 30µl PCR reaction-mix (15 µL of Promega GoTaq® Colorless Master Mix, 0.75 µL of each 5ng/uL primer, 1 µL of 15ng/µL DNA, and 10.5 µL IDT® nuclease-free water). All reactions were performed in a 2720 Thermocycler with PCR conditions optimized for each DNA locus (Sup. Mat. I). PCR products were purified and sequenced by Macrogen, Inc. (Seoul, South Korea) with the same primers used in amplifications.

Phylogenetic inference

All sequences were aligned in CLC sequencer Viewer 6 software (CLC BIO) using the Clustal W algorithm. Nuclear and chloroplastic regions were first analyzed separately to check for possible incongruence in tree topology and, in the absence of incongruences, we concatenated them and performed a total evidence approach. Phylogenetic relationships were inferred using Bayesian (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP) approaches.

Bayesian inferences were done applying a Markov-Chain Monte Carlo (MCMC), as implemented in BEAST version 1.7.4 (Drummond et al. 2012). The analysis was partitioned using the best models of evolution inferred by MrModeltest 2.3 (Nylander

2004) and using the Akaike Information Criterion (AIC). The Hasegawa-Kishino-Yano (HKY85) substitution model was the best model for all genes. We performed four independent runs of 10,000,000 generations with four chains (one cold and three heated), and sampling trees every 1,000 generations. The diagnostic of convergence was performed in Tracer version 1.7 (Rambaut & Drummond 2007) and all runs were combined in LogCombiner version 1.7.4 (Drummond et al. 2012). The first 10% of the runs was discarded in TreeAnnotator version 1.7.4 (Drummond et al. 2012) and the remaining trees (36,000) were used to calculate the maximum credibility tree.

The partitioned ML analysis was performed using RaxML version 7.4.4 (Stamatakis 2006) and run in the Cipres Science Gateway (<http://www.phylo.org/portal2>) using the GTRGAMMA model for each DNA partition. Node support was evaluated by a bootstrap analysis using 10,000 replicates following the search of the best-scoring ML tree (Felsenstein 1985).

Finally, the same data set was analyzed using the parsimony criterion in PAUP* version 4.0b10 (Swofford 2002). Heuristic searches were carried out using Tree-Bisection-Reconnection (TBR) branch swapping. A majority-rule consensus tree was calculated from the most parsimonious trees and bootstrap support for the nodes was computed by 1000 replicates (Felsenstein 1985). The topology congruence of the trees inferred by BI, ML and MP was evaluated by visual inspection, comparing the topology and branch supports.

Trait evolution

In order to determine if the evolution of traits in our plant group was affected by the phylogenetic relationships, we tested the hypothesis of phylogenetic signal of traits in

BayesTraits (Pagel & Meade 2006). In doing so, we ran four MCMC searches for 10,000,000 generations; sampling trees every 1,000 generations. To determine the phylogenetic signal of morphological traits, the lambda parameter (λ) was estimated. This parameter measures the degree to which the covariance between species traits agrees with the expectation based on a given model of evolution (Freckleton et al. 2003). Here, $\lambda = 0$ suggests that traits vary independently of the phylogeny, while $\lambda = 1$ indicates that the phylogenetic history of the group has a strong effect on trait evolution (Pagel 1997, Pagel 1999). We evaluated the type of evolutionary model (Random or Directional) that matched better the data through the comparison of Bayes Factors (BF), as implemented in Tracer version 1.5 (Drummond et al. 2012).

Plant and insect trait correlation

To properly test the effect of the phylogeny vs. the pollinator environment on the evolution of the floral traits, we wanted to identify any possible correlation between plant and pollinator traits. To do this, we constructed a correlation matrix using the partial Pearson's correlation coefficient based on the full morphological dataset. All statistical analyses were performed on log-transformed data and Bonferroni correction was used to control for multiple testing using R package version 1.4 (available in <http://www.R-project.org>).

RESULTS

Morphological traits

In terms of corolla traits, *C. crenatiflora* was the species with the largest corolla size (CA) and distance between the reproductive structures and elaiophore (DSE and DAE), while *C.*

purpurea was the smallest one (Table 1). The size of the elaiophore (EA) was the trait with the highest variability among species (range: 0.04 - 0.6 cm²); with *C. purpurea* displaying the smallest gland area, and *C. filicaulis* spp. *luxurians* the largest one (which corresponded approximately to the 50% of its corolla area; Table 1).

Pollination was performed only by Hymenoptera, which for the purposes of this study may be classified as oil- or pollen-collecting. The known genera of oil-collecting bees (*Centris* and *Chalepogenus*) were responsible of the pollination of seven out of nine *Calceolaria* species (Table 2). *Calceolaria arachnoidea*, *C. corymbosa*, *C. filicaulis* spp. *luxurians* and *C. paralia* were principally pollinated by *Centris nigerrima*, while, *C. filicaulis*, *C. purpurea* and *C. valdiviana* were pollinated by *Chalepogenus* sp. Finally, *Calceolaria biflora*, *C. cana* and *C. crenatiflora* were exclusively visited by pollen-collecting bees: *Megachile semirufa*, *Anthidium* sp. and *Bombus terrestris*, respectively. In general, most of the pollinator traits were larger in *Centris* than in *Chalepogenus*. As expected, the structures responsible of flower handling and oil collection (MOC and LOZ) were larger in *Centris* than in *Chalepogenus* (Table 2). All pollen-collecting bees had a size similar to *Centris*, with *Bombus terrestris* and *Megachile semirufa* having the largest values and *Anthidium* sp. the smallest one.

Phylogenetic inferences

No major incongruences were found among nuclear and chloroplastic partitions, and the total evidence approach (3509 nucleotides) provided a highly resolved tree. The three phylogenetic reconstructions resulting from the total evidence analysis with BI, ML and MP approaches depicted congruent but differently resolved topologies, with the best



resolution obtained with the BI approach (Figure 3).

The Bayesian maximum credibility tree showed strong support ($\geq 95\%$ BPP) in most nodes, defining one clade (clade III; Figure 3) and two external taxa (I and II; Figure 3). *Calceolaria biflora* is the most basal species (taxon I), followed by *C. paralia* (taxon II). Clade III is polytomized and consists of two subclades (IIIA and IIIB) and two taxa (IIIC). Subclade IIIA contained *C. filicaulis* spp. *luxurians* positioned as a sister species to a well-resolved clade containing *C. corymbosa* and *C. crenatiflora*. Subclade IIIB strongly supports *C. arachnoidea*, which is basal and sister species of a well-resolved clade, composed by *C. cana* and *C. valdiviana*. Finally, IIIC consisted of two polytomized species: *C. purpurea* and *C. filicaulis*.

Trait evolution

The analysis of the mode of evolution of floral traits showed that they have all been evolving following a Random Walk model of evolution (Table 3). The phylogenetic signal analysis reported small and non-significant λ values for four out of five floral traits, as calculated in the Bayes Factor estimation (Table 3). Corolla size (CA) was the only character that showed a λ value significantly different from zero. However, the low λ value for this trait (0.4), suggests that the phylogenetic history of the group has a minimal effect on the evolution of this trait (Pagel 1999).

Plant and pollinator trait correlation

Overall, the partial Pearson's correlation matrix showed that two of five floral traits were

positively and significantly correlated among them (DSE vs DAE, $r=0.88$, $P<0.001$), while for pollinator traits only thorax width (TW) and maximum extension for oil collection (MOC) was correlated, however its significance was lost after Bonferroni correction (Table 4). In the same way, in terms of the relationship between floral and pollinator traits, herkogamy (H) was the only trait that showed a significant correlation with two pollinator traits (TL and LOZ), but its significance could not be retained after Bonferroni correction (Table 4).

DISCUSSION

The main goal of this study was to evaluate the importance of the phylogenetic history and ecological environment (pollinators) on the morphological variation in *Calceolaria* species. To analyze the effect of the phylogeny on plant trait variation, we reconstructed the phylogenetic relationships of nine *Calceolaria* species and evaluated the phylogenetic signal. Afterward, and to quantify the effects of the ecological environment on the evolution of floral traits, we explored the covariation between floral and insect traits in the same nine *Calceolaria* species. Our results showed that the phylogenetic history played a relatively minor role in the evolution of floral traits. Indeed, we did not observe any phylogenetic signal in most traits under study (with the exception of a low signal detected in corolla size). Contrary to our expectations, non-structural relationship was found between floral traits and pollinator morphology of the nine *Calceolaria* species.

Is floral trait evolution phylogenetically restricted in *Calceolaria*?

Phylogenetic relationships in the subgenus Rosula

In this study, we reconstructed the phylogenetic relationship of nine *Calceolaria* species of the subgenus *Rosula*, incorporating five new species whose relationships were unknown until today. For this, we used six genes (two nuclear and four chloroplastic), which, as a whole, contributed to inferring a more complete phylogeny for the subgenus.

Our Bayesian phylogenetic inference supported two external taxa (I and II) and one major clade (Clade III, Figure 3). The position of the most basal taxon (*C. biflora*, taxon I) coincides with previous studies (Anderson 2006, Cosacov et al. 2009), while the never phylogenetically studied *C. paralia* (taxon II) appeared to be external to Clade III. The main clade (III) contained the remaining eight species and was formed by a group of polytomized subclades/taxa. A well-resolved subclade (IIIA) included the basal *C. filicaulis* spp. *luxurians*, *C. crenatiflora* and *C. corymbosa*. These relationships coincide with those established by Cosacov et al. (2009), and from this perspective our study is not incongruent with previous investigations. The well-supported subclade IIIB was composed by *C. cana* and *C. valdiviana* as sister species of the external *C. arachnoidea*. Since their relationships had never been inferred prior to this study, this result contributes to the understanding of the relationships between species in the genus. Taxa IIIC (*C. filicaulis* and *C. purpurea*) were polytomized, indicating that more variable markers should be used to resolve this part of the phylogeny. However, the fact that we did not recover *C. filicaulis* as sister to *C. filicaulis* spp. *luxurians* supports the idea proposed by Anderson (2006) and Cosacov et al. (2009), which proposes that these species should be considered as different taxa (Figure 3).

Although increasing the number of molecular markers (from two in other studies, to six in this investigation) did not allow us to completely resolve a tree for the group, our

phylogenetic inferences represent an advance in the understanding of the species relationships in this genus. However, the difficulties encountered in the tree reconstruction should be considered in further studies, since this shows that if a well-resolved phylogeny is to be obtained for the genus, many more markers than the ones used here should be included.

Trait evolution

The absence of phylogenetic signal in most of the study floral traits, excepting for corolla size, seems to be common in some plant species reflecting the labile nature of some floral traits and pollinator groups under some circumstances (Prather 1999, Pérez et al. 2006). Indeed, floral trait lability has been demonstrated in different plant-pollinator systems such as *Gladiolus* (Valente et al. 2012), *Disa* (Johnson et al. 1998), *Aquilegia* (Whittall & Hodges 2007) or *Costus* (Kay et al. 2005). Nevertheless, the majority of these studies have been performed in systems involving shifts of pollinator functional groups (e.g., from hummingbird to bat pollination; but see Armbruster & Balwin 1998). Our study is to our knowledge the first to indicate that such processes may also apply to systems involving similar pollinator groups. Respecting of the detection of a lower phylogenetic signal on corolla size evolution is not surprising. Indeed, this has been also observed in other plant species (see Blomberg et al. 2003). In general, it has been suggested that corolla size evolve in a more conservative way than other floral traits (e.g., sexual traits) due to their origin and/or their function during pollination (Alcantara & Lohmann 2011). For example, the evolution of overall flower size may be a result of a common developmental pathway between the external floral parts (Conner & Sterling 1995, Diggle 2002) or, and from an

adaptive point of view, may be related to the attraction of a particular pollinator group (*e.g.*, specialized species; Alcantara & Lohmann 2011). However, due to that in our study the phylogenetic signal on corolla size was minimal, unlike other study systems, more study is need to achieve the proximal causes of our pattern.

Is the ecological environment driving the evolution of floral traits in *Calceolaria*?

The evolution of floral traits can be restricted and guided by the ecological environment, as for example pollinator types (Fenster et al. 2004), or by the historical relationships of the studied group (Alcantara & Lohmann 2011). In this part of the study, we wanted to quantify the correlation between pollinator type and morphology, and floral traits important to the interaction. Such an analysis would allow us to identify floral traits, which might be evolving in response to pollinators.

Our results showed that only the distance between reproductive structures and the oil reward (DSE vs DAE) were positively correlated (Table 5). This would indicate that these two traits appear to be evolving together, what could be explained by their key function during the pollination process. Indeed, these might be important characters defining the precision of cross-pollination (Armbruster et al. 2004, Nattero et al. 2010). Regarding the pollinator traits, the absence of correlation between them may be explained in part, if bees respond to small variations in the position of the oil gland. For example, it is likely that insect responses depend on morphological adjustments such as: a) an increase in foreleg and body size and/or, b) an elongated foreleg keeping constant body size. While in case a) we expect a strong correlation between body and foreleg size, the absence of such a correlation would support the second hypothesis (Steiner & Whitehead 1990).

The analysis of the structural relationship between plants and pollinators showed that all the studied traits were not correlated. However, the absence of association does not imply that pollinators play no role in the floral evolution of this plant group. For instance, the absence of a trait-pollinator correlation suggests that the origin of *Calceolaria* species evolved simultaneously with the establishment of the oil-rewarding interaction (Molau 1988, Sérsic 2004, Cosacov et al. 2009) in the late Tertiary (6-1 Myr, Renner & Schaefer 2010). Under such historical scenario, the oil-rewarding relationship may have evolved only recently, and plants may be still in the process of adjustment to the specialized pollination. One way to inquire into this question is to consider additional floral and pollinator traits, such as corolla aperture and width relative to pollinator size, and quantity and quality of floral reward among others. To determine the extent to which results from this study truly reflect an evolutionary process that is still in course, comparable studies should be performed in *Calceolaria* species not examined in this study.

In summary, at the light of these results we conclude that historical relationships between species belonging to the subgenus *Rosula* play a minor role in the evolution of the corolla traits including in this investigation, specially in those involving in floral specialization (*i.e.*, herkogamy and gland area). Accordantly with this, the absent of phylogenetic signal suggest that floral evolution in *Calceolaria* could be responding to the ecological contingency. Unfortunately, the absent of correlation between floral and pollinator traits does not allow us to assert that pollinator are the main ecological force acting in the evolution of *Calceolaria* traits. However, the lasted could not rule out particularly because the relationship between *Calceolaria* and its pollinators has been described as one of the youngest oil-secreting flowers specialized interaction (Renner &

Schaefer 2010); therefore species could be still in early states of the plant-pollinator morphological adjustment. In this context, greater efforts must be made in terms of taxa sampling and detection of key floral traits that could be mediating the relationship between *Calceolaria* species and its pollinators, all of which allow us to approach the proximal and ultimate factors shaping the floral trait evolution in this specialized pollination system.

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TABLES

Table 1. Geographic coordinates, floral traits and principal pollinator of the sampled *Calceolaria* species. Corolla area (CA), herkogamy (H), distance stigma-elaiophore (DSE), distance anthers-elaiophore (DAE), and elaiophore area (EA).

Species	Coordinates	CA	H	DSE	DAE	EA	Pollinator species
<i>C. arachnoidea</i>	36°36' S / 72°00' W	4.71	0.98	0.78	0.77	0.06	<i>Centris nigerrima</i>
<i>C. biflora</i>	50°52' S / 72°44' W	4.32	0.04	1.39	1.18	0.51	<i>Megachiles semirufa</i>
<i>C. cana</i>	35°35' S / 70°00' W	1.20	0.07	0.64	0.61	0.06	<i>Anthidium</i> sp.
<i>C. corymbosa</i>	37°46' S / 72°47' W	1.50	0.10	1.10	1.01	0.08	<i>Centris nigerrima</i>
<i>C. crenatiflora</i>	38°28' S / 71°38' W	5.61	0.10	1.52	1.30	0.14	<i>Bombus terrestris</i>
<i>C. filicaulis</i>	36°36' S / 72°00' W	1.07	0.57	0.59	0.51	0.28	<i>Chalepogenus</i> sp.
<i>C. filicaulis</i> spp. <i>luxurians</i>	33°19' S / 70°16' W	2.09	0.10	0.96	0.86	0.60	<i>Centris nigerrima</i>
<i>C. paralia</i>	35°02' S / 70°36' W	1.71	0.16	0.98	1.07	0.19	<i>Centris nigerrima</i>
<i>C. purpurea</i>	33°23' S / 70°27' W	0.60	0.05	0.31	0.44	0.04	<i>Chalepogenus</i> sp.
<i>C. valdiviana</i>	37°49' S / 72°57' W	1.37	0.08	0.58	0.71	0.09	<i>Chalepogenus</i> sp.

Table 2. Mean \pm standard deviation values for the traits measured on the principal pollinators of the sampled *Calceolaria* species. Sample size (n), Total length (TL), Thorax width (TW), Maximum extension for oil collection (MOC) and Length of the oil-collecting zone (LOZ).

Pollinator type	Plant species	n	TL	TW	MOC	LOZ
<i>Centris</i>	<i>C. arachnoidea</i>	60	1.57 \pm 0.06	0.73 \pm 0.02	1.75 \pm 0.06	0.68 \pm 0.05
	<i>C. corymbosa</i>	3	1.39 \pm 0.02	0.60 \pm 0.01	1.72 \pm 0.18	0.53 \pm 0.06
	<i>C. filicaulis</i> spp. <i>luxurians</i>	4	1.60 \pm 0.04	0.72 \pm 0.02	1.67 \pm 0.04	0.60 \pm 0.01
	<i>C. paralia</i>	2	1.54 \pm 0.02	0.69 \pm 0.03	1.80 \pm 0.09	0.55 \pm 0.04
<i>Chalepogenus</i>	<i>C. filicaulis</i>	6	0.96 \pm 0.05	0.37 \pm 0.02	0.94 \pm 0.04	0.14 \pm 0.01
	<i>C. purpurea</i>	5	0.57 \pm 0.12	0.39 \pm 0.10	0.84 \pm 0.28	0.13 \pm 0.61
	<i>C. valdiviana</i>	1	1.04	0.35	0.89	0.16
Pollen bees	<i>C. biflora</i>	10	1.46 \pm 0.17	0.71 \pm 0.03	1.65 \pm 0.08	0
	<i>C. cana</i>	3	0.94 \pm 0.07	0.45 \pm 0.01	0.97 \pm 0.01	0
	<i>C. crenatiflora</i>	2	1.69 \pm 0.22	0.89 \pm 0.23	5.52 \pm 1.16	0

Table 3. Phylogenetic analysis of the best model of evolution (Random Walk v/s Directional) and phylogenetic signal of each floral trait in the *Calceolaria* species here studied. Corolla area (CA), herkogamy (H), distance stigma-elaiophore (DSE), distance anthers-elaiophore (DAA), and elaiophore area (EA). Values are log-Bayes Factor \pm standard error. Bold numbers indicate significant differences at $p < 0.05$.

	Random v/s Directional	Phylogenetic signal
CA	0.02 \pm 0.05	0.40\pm0.03
H	0.04 \pm 0.05	0.30 \pm 0.04
DEE	0.02 \pm 0.05	0.13 \pm 0.05
DAE	0.05 \pm 0.05	0.19 \pm 0.05
EA	0.001 \pm 0.05	0.001 \pm 0.04

Table 4. Partial Pearson's correlation matrix among mean floral traits and mean pollinator traits for the study *Calceolaria* species. Floral traits: Corolla area (CA), herkogamy (H), distance stigma-elaiophore (DSE), distance anthers-elaiophore (DAE), and elaiophore area (EA). Pollinator traits: total length (TL), thorax width (TW), maximum extension for oil collection (MOC) and length of the oil-collecting zone (LOZ). Bold numbers indicate significant correlations at $p < 0.05$ that remains after Bonferroni correction.

	Floral traits				Pollinator traits			
	H	DSE	DAE	EA	TL	TW	MOC	LOZ
CA	0.64	0.24	0.18	-0.04	-0.12	0.39	0.36	-0.53
H		0.08	-0.58	-0.13	0.74	0.19	-0.13	0.74
DSE			0.88	0.47	-0.1	0.01	0.33	-0.46
DAE				-0.38	0.66	0.29	-0.06	0.64
EA					0.49	0.14	-0.46	0.37
TL						0.59	-0.11	0.39
TW							0.65	0.19
MOC								-0.52

FIGURES

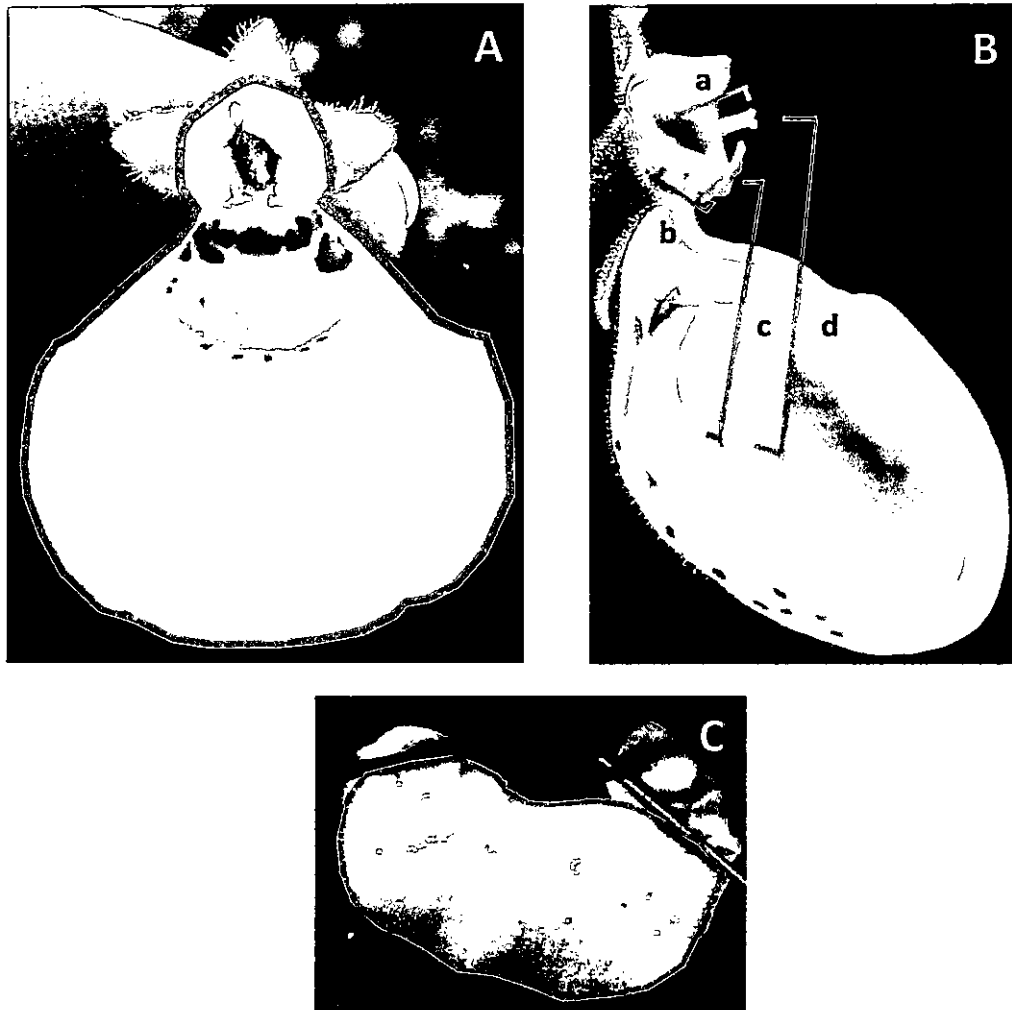


Figure 1. Floral traits measured from digital pictures of *Calceolaria*. A) corolla area, B) measures of reproductive structures (a: stigma length; b: anther length) and distance to elaiophore (c: distance from stigma to elaiophore (DSE); d: distance from anther to elaiophore, (DAE)) and, C) area of the elaiophore (EA).

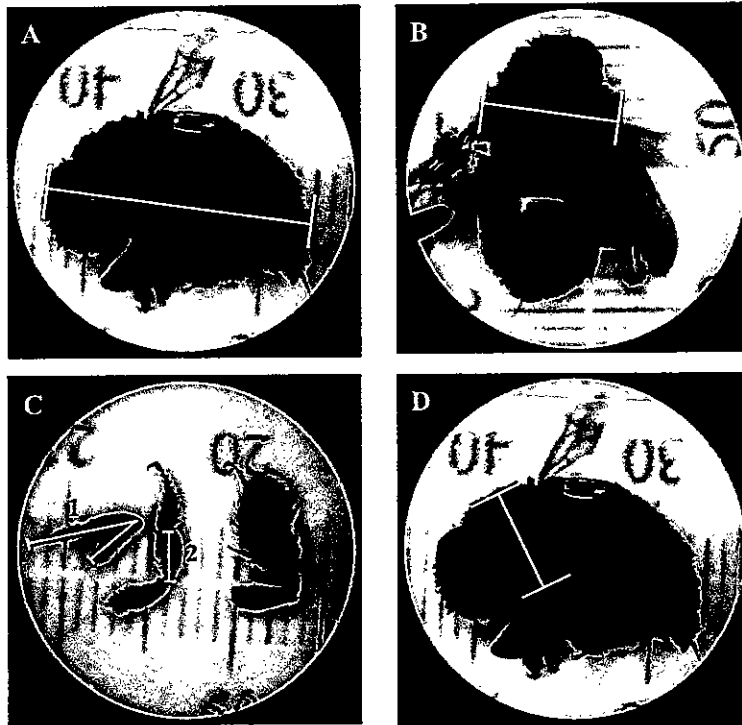


Figure 2. Pollinator traits measured from digital pictures. A) Total length (TL), B) thorax width (TW), C) leg length (1) and length of the oil collecting zone (2) (LOZ) and, D) thorax height (TH).

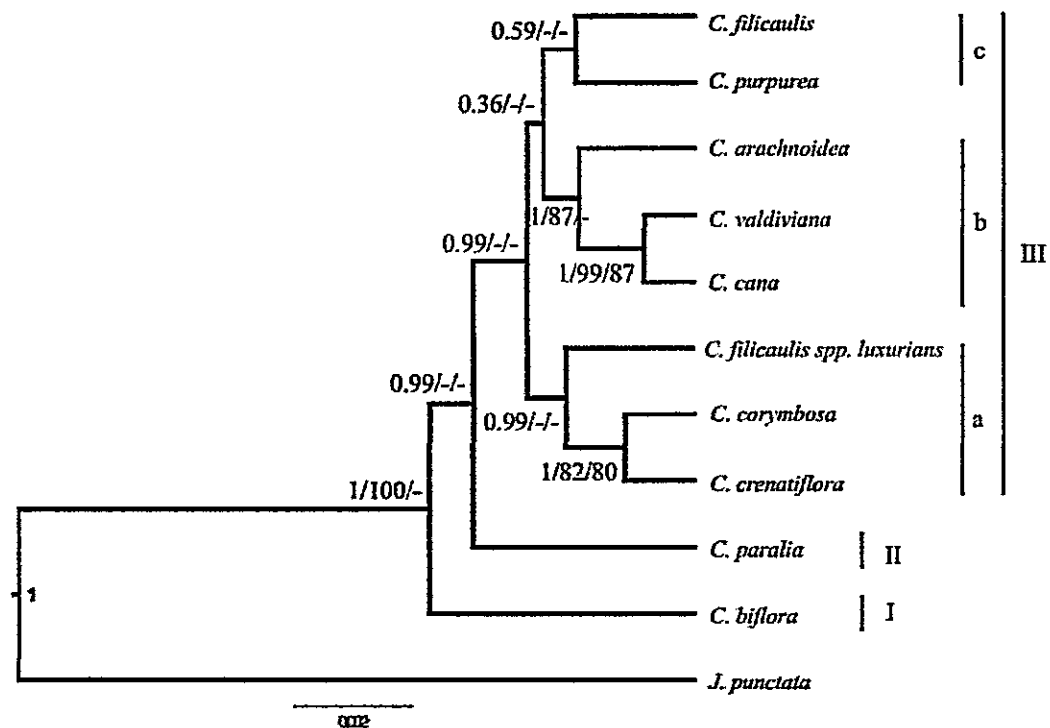


Figure 3. Phylogenetic relationships among *Calceolaria* species resulting from the total evidence. Values above each node correspond to posterior probability/bootstrap support under Bayesian, Maximum Likelihood and Maximum Parsimony inference, respectively. Vertical bars represent main groups: taxa (I and II), subclade III and sub-clades A, B and C.

SUPPLEMENTARY MATERIAL

Table S1. Primers used for amplifying and sequencing.

Primer name	Direction	Primer sequence 5'-3'	Source
ITS spacer+5.8S			
ITS2	Forward	ITS 2: GCTGCGTTCTTCATCGAT	White et al. 1990
ITS5	Reverse	ITS 5: GGAAGTAAAAGTCGTAACAAGG	
ITS3	Forward	ITS 3: GCATCGATGAACGCAGC	White et al. 1990
ITS4	Reverse	ITS 4: TCCTCCGCTTATTGATAT	
matK			
323f	Forward	323f: ATTNTCAAATCNTAKCAGAGG	Andersson 2006
1189r	Reverse	1189r: CGGCTTACTAATRGGATGC	
rpl32			
ndHf	Forward	ndHf: CTGCTTCCTAAGAGCAGCGT	Shaw et al. 2007
rpl32r	Reverse	rpl32r: CAGTTCCAA AA A AACGTACTTC	
rpl32f	Forward	rpl32f: CAGTTCCAAAAAACGTACTTC	Shaw et al. 2007
trnL	Reverse	trnL: GGAAGTAAAAGTCGTAACAAGG	
trN			
trnS	Forward	trnS: AACTCGTACAACGGATTAGCAATC	Shaw et al. 2007
trnG	Reverse	trnG: GAATCGAACCCGCATCGTTAG	

Table S2. PCR profiles for DNA amplification: (1) initial denaturation; (2) number of cycles; (3) denaturation, annealing, and elongation steps for each cycle; (4) final elongation step. Temperature and duration are indicated for each step. Asterisk represents a ramp of 0.3°C per second until 65°C.

Locus	PCR profiles
ITS/matK	(1) 94 °C/5 min; (2) 25; (3) 94°C/1 min; 56°C /30 s; 72°C/ 45 s; (4) 72°C /10 min
rpl32/trN	(1) 80°C/5 min; (2) 30; (3) 90°C/1 min; 50°C /1 min*; 65°C/ 4 min; (4) 65°C /5 min

CAPITULO III

ASSESSMENT OF REPRODUCTIVE BARRIERS IN TWO SYMPATRIC OIL-REWARDING *CALCEOLARIA*

ABSTRACT

Reproductive isolation in plants is not the effect of a single barrier; instead is a consequence of a cumulative effect of different pre- and post-zygotic barriers. Moreover, because in some cases a given barrier can reduce, but not totally preclude, the interspecific gene flow, quantifying the strength of different isolation barriers is necessary to clarify their role in the speciation process. Most of the studies have been developed in species with generalized pollination systems. However, patterns of reproductive isolation may be different depending of the pollination system, particularly in specialized systems, because specific associations with pollinators may be changing the relative importance of pre and post-zygotic mechanisms. *Calceolaria* is a highly diversified Andean genus composed by flowers displaying nonvolatile oils used to reward a group of solitary oil-collecting bees. In order to determine the factors responsible of the maintenance of species borders in sympatry, we estimated the strength of different reproductive barriers in two *Calceolaria* species. For this propose, we estimated three pre-zygotic barriers: two in the field (microhabitat and pollination isolation) and one in the greenhouse (intra and interspecific cross-pollination) and, calculated the seed germination of parental and hybrid as a measure

of offspring viability (post-zygotic isolation). Our results showed that these species are highly isolated by geography, the identity of their principal pollinators and post-pollination mechanism. However, hybrids inviability was not detected, what suggests a minor contribution of post-zygotic barriers, compare to pre-zygotic ones, to the total reproductive isolation of these species.

KEYWORDS: *Calceolaria*, hybrids, isolation barriers, oil-collecting bees, specialization, speciation.

INTRODUCTION

Reproductive isolation is a key component of the speciation process because it disrupts gene flow between interbreeding populations (Coyne & Orr 1998). It has long been demonstrated that several pre- and post-zygotic mechanisms lead to reduction of inter-specific gene flow, which operate at many stages of an organism's life cycle (Dobzhansky 1937). For example, in plants, pre-zygotic mechanisms such as spatial isolation, pollinator types and physical pollen incompatibility can prevent cross-pollination among different species before fertilization (Yost & Kay 2009). When fertilization has occurred, post-zygotic barriers, as hybrid sterility or seed unviability can inhibit the establishment and spread of the new offspring (Coyne & Orr 2004).

Reproductive isolation in plants is rarely the effect of a single barrier; instead species reproductive isolation is a consequence of a cumulative effect of different pre- and post-zygotic barriers (Coyne & Orr 2004). Moreover, because in some cases a given barrier can reduce, but not totally preclude, potential interspecific gene flow, quantifying the strength of different isolation barriers is necessary to properly clarify their role in the speciation process (Widmer et al. 2009). Despite the increasing interest in exploring this topic, only a few studies have estimated the magnitude of different isolating barriers on interspecific reproductive isolation (see review in Lowry et al. 2011). For example, in a study of seven barriers of isolation in sympatric *Costus* species, Kay (2006) reported high values of reproductive isolation index (*RI*) for mechanical and post-pollination factors and low values for offspring viability and F_1 male sterility, suggesting that pre-zygotic barriers contribute more than post-zygotic ones to total isolation. In the same way, a study of nine isolation

barriers in *Mimulus lewisii* and *M. cardinalis*, Ramsey et al. (2003) reported that habitat and pollination isolation exhibit higher *RI* values than F_1 seed germination and F_1 survival, showing again that pre-zygotic mechanisms are the most important reproductive barriers to gene flow in the studied species.

In general terms, most studies in plants aimed to estimate the relative importance of different mechanisms of reproductive isolation suggest that pre-zygotic barriers are stronger than post-zygotic ones (Nossil et al. 2005). However, the generality of these patterns needs to be confirmed as some evidence indicates the opposite (Winder et al. 2009). For example, Mediterranean orchids sharing pollinators have more divergent karyotypes than species pollinated by different floral visitors, suggesting that post-pollination barriers play an important isolation role in groups that have non-specific pollinators (Cozzolino et al. 2004). Likewise, in a study of *Dactylorrhiza* species, De hert et al. (2012) found that seeds produced by interspecific crosses exhibit low hybrid viability, concluding that post-zygotic barriers prevent the breakdown of the species, despite hybridization is frequently observed in the field.

Most studies addressing the evolution of reproductive isolation in plants have been developed in species with generalized pollination systems, such as *Mimulus* (Martin & Willis 2007) and *Chamaecrista* (Costa et al. 2007) (but see Diaz et al. 2006, Scopece et al. 2007). However, patterns of reproductive isolation may differ depending on the pollination system, particularly in specialized systems, because specific associations with pollinators may change the relative importance of pre and post-zygotic mechanisms (Cozzolino & Scopece 2008). Such could be thus the case of *Calceolaria* L. (Calceolariaceae) and its oil-collecting pollinators (Sérsic 2004). *Calceolaria* is a highly diversified Andean genus

whose flowers present nonvolatile oils used as reward by a group of solitary oil-collecting bees (Rasmussen & Olessen 2000, Sérsic 2004). An extensive study performed by Molau (1988) suggested that some *Calceolaria* species are reproductively isolated by pollinators, geographic range, and habitat preference. However, a study performed in sympatric *C. uniflora* and *C. polyrhiza* documented the presence of intermediate phenotypes and suggested the possibility of natural hybridization (Sérsic et al. 2001). It is then surprising that even if hybridization has been observed, the biological mechanisms responsible of reproductive isolation in *Calceolaria* have not been identified.

Here we estimate different barriers of reproductive isolation in two sympatric, but morphologically divergent, *Calceolaria* species (*C. arachnoidea* and *C. filicaulis*). Specifically, we address the following questions: a) Do pre- and post-zygotic reproductive barriers exist between species? b) if so, what is their strength and relative importance?. To answer these questions we quantify pre-zygotic barriers (microhabitat and pollination isolation) in the field, and perform intra- and interspecific pollination crosses in the greenhouse. In addition, we examine germination of parental and hybrid seeds as a measure of offspring viability (post-zygotic isolation). We finally use these data to calculate reproductive isolation indexes.

MATERIALS AND METHODS

Species and study site

Calceolaria filicaulis spp. *filicaulis* (hereafter *C. filicaulis*) and *C. arachnoidea* are closely related perennial herbs (see Results, in Chapter I) that grow close to streams and rivers in

the high Andes of Chile. Except for some specific localities in central Chile, the two species have ranges that in general do not overlap (Ehrhart 2000). As all *Calceolaria* species, *C. filicaulis* and *C. arachnoidea* have zygomorphic flowers composed by 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 several morphological traits, especially those related to corolla characteristics and reproduction (Figure 1). On the one hand, the strictly allogamous *C. filicaulis* is visited by *Chalepogenus* bees (M. Murúa, unpub. data). This species has a small yellow corolla with lobes spaced apart and the oil gland located close to the corolla aperture, that allows easy access to pollinators. On the other hand, the partially allogamous *C. arachnoidea* is visited by *Centris* bees (M. Murúa, unpub. data) and displays a large purple corolla, with lobes tightly closed and the oil gland in a hardly accessible position, making oil collection more difficult. Hybrids often exhibit intermediate phenotypes respect to their putative parental species (Figure 2). Generally, flowers have a reduced yellow upper lobe and an inflated red lower lobe. Plants are taller than *C. filicaulis* but smaller than *C. arachnoidea* (mean \pm SE; *C. filicaulis*: 20.14 \pm 0.20 cm.; Hybrids: 26.2 \pm 11.72 cm.; *C. arachnoidea*: 28.5 \pm 10.81 cm.). The same is observed for floral display (mean \pm EE; *C. filicaulis*: 2.26 \pm 0.11 flower/plant; Hybrids: 4.4 \pm 1.97 flower/plant; *C. arachnoidea*: 6.58 \pm 0.29 flower/plant) and for other corolla traits (Figure 2). Respect to pollination ecology, is unknown the species responsible of the pollination of hybrids, however in a preliminary study in five consecutive days of pollinator census no insects were observed visiting these flowers (M. Murúa, unpub. data).

This study was conducted during the summer season of 2012 at Altos de Lircay National Reserve (35°36'S, 71°00'W, 2200 m elevation) in Chile. The site has a

Mediterranean climate, with most rainfalls concentrated in the winter season (Di Castri & Hajek 1976). In the site, *C. filicaulis* and *C. arachnoidea* are sympatric and distributed in monospecific patches, surrounded by a vegetation dominated by *Mimulus luteus* and *M. cupreus* (Schrophulareaceae), *Hypochoeris acaulis* (Asteraceae), *Pozoa coriacea* (Apiaceae) and *Azorella incisa* (Apiaceae). Despite hybrids between these two *Calceolaria* species are frequently described in nature (Ehrhart 2000), there are found in low abundance inside the Reserve (*i.e.*, 4% of the total sampled population $n=342$).

1. Pre-zygotic barriers

1.1 Microgeographic isolation

To determine whether the spatial structure contributes to the isolation between *C. filicaulis* and *C. arachnoidea*, we recorded the coordinates of each plant in a 2500 m transect. In total, 160 plants of *C. filicaulis* and 168 of *C. arachnoidea* were geo-referenced. Following the methodology described by Ramsey et al. 2003, we first defined a grid covering the studied area, and afterwards a fixed number of quadrants was randomly sampled 1000 times. The presence of one or the two species in a single quadrant was recorded after randomization, which permitted classification of homospecific or heterospecific quadrants. Finally, we counted the number of each type of quadrant and used this value to estimate the strength of the microgeographical reproductive barrier (see below). Given that this type of analysis is sensitive to quadrant size, we performed the procedure using grids with different quadrant sizes (20m x 20m and 50m x 50m). The complete procedure was performed in ArcMap 10.1 and ModelBuilder (ESRI, CA). The microgeographical isolation index (*RI* *microgeographical*) was expressed as:

$$RI_{microgeographical} = 1 - \left(\frac{\#heterospecific\ quadrants}{\#heterospecific\ quadrants + \#homospecific\ quadrants} \right)$$

The index provides values that range from 0 (complete overlap) to 1 (complete isolation).

1.2 Floral visitors

We recorded all floral visitors in both *Calceolaria* species during January 2012. Focal observations of 15 minutes per plant were performed during six sunny days from 09:00 to 18:00 hours (50 hours of observation per species). Only pollinator species that touched the reproductive structures of the plant were considered as legitimate pollinators. A sample of every pollinator species observed in the field was 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 ($RI_{pollinator}$) expressed as:

$$RI_{pollinator} = 1 - \text{proportion of visitation rate composed by a shared pollinator}$$

This index gives values ranging from 0 (no isolation) to 1 (complete isolation).

1.3 Post-pollination isolation

We assessed the level and symmetry of post-pollination isolation through a cross-pollination experiment. For this, we grew plants of both species in the greenhouse, using seeds from 40 fruits of each species, previously collected during the field season. Twenty

seeds per fruit were chosen and sown individually in seeding pots (1000 seeds per species). Seeds were germinated with a protocol of daily watering and constant temperature of 18°C (Erwin 1994). After three weeks, plants were transplanted into individual growing pots until flowering. Then, 45 plants per species were randomly assigned to one of the following treatments: 1) *C. filicaulis* intraspecific cross-pollination; 2) interspecific pollination with *C. archnoidea* as pollen donor species; 3) *C. archnoidea* intraspecific cross-pollination and 4) interspecific pollination with *C. filicaulis* as pollen donor species. For each treatment, we randomly selected two plants, one as pollen donor and the other as pollen receiver. For all treatments, three buds of the receiver plant were emasculated and pollinated with pollen of three flowers of the donor plant. Fruits were left intact to develop and, once fully ripe, they were collected and the seeds counted. The mean seed set of each treatment was compared using Wilcoxon paired signed rank tests, using R version 2.15 (<http://www.R-project.org>). Finally, the reproductive post-pollination isolation index ($RI_{postpollination}$) was estimated as:

$$RI_{postpollination} = 1 - \left(\frac{\#seeds/inter-specific\ pollination}{\#seeds/intra-specific\ pollination} \right)$$

Where $RI = 0$ indicates no isolation, while 1 represents complete post-pollination isolation.

2. Post-zygotic isolation

2.1 Seed germination

To determine reproductive isolation due to differences in the germination success of

hybrids, we carried out a germination experiment in the greenhouse. For this, we chose 10 seeds of each fruit produced in the post-pollination treatments (see above) and sew them in individual seeding pots. Seeds were grown at constant temperature (18°C). After seed germination, the proportion of germinated seeds was estimated in all treatments. The proportion of germinated seeds belonging to intra- and interspecific pollination treatments were then compared using Wilcoxon paired signed rank tests in R package. Finally, we calculated the post-zygotic isolation index, which quantifies the magnitude of post-zygotic isolation through seed germination ($RI_{viability}$):

$$RI_{viability} = 1 - \left(\frac{\text{proportion of seed germination}}{\text{proportion of parental germination}} \right)$$

Where RI indicates complete hybrid viability (no isolation) and 1 represents total isolation.

Confidence intervals for all isolation indexes were estimated by bootstrapping 1000 times each estimated indexes in R package.

RESULTS

1. Pre-zygotic isolation

1.1 Microgeographic isolation

The analysis of micro-geographic isolation indicated, as expected, that the frequency of heterospecific quadrants increased with quadrant size. However, the estimation of micro-



geographical index did not show important differences for the two quadrant sizes (20x20 m²: 0.86 ± 0.06; 50x50 m²: 0.76 ± 0.06). As pollinators were observed flying long distances (pers. obs.), we used only the results from grids with 50x50 quadrants. Therefore, after 1000 replicates the calculated mean $RI_{microgeographical}$ was 0.76 ± 0.06 (Table 1).

1.2 Floral visitors

We only observed Hymenoptera visiting the two species, even if their number and identities differed for each of the plant species (Table 2). While *C. filicaulis* was visited only by two species (*Chalepogenus caeruleus* and *Bombus terrestris*), with *Chalepogenus caeruleus* performing 82% of the visits, *C. arachnoidea* was visited by four different bee species, with the principal pollinator being *Centris nigerrima* (86% of visits). *Bombus terrestris* was the only species shared between both *Calceolaria* species, performing similar visitation rates in both plant species (Table 2). The estimation of $RI_{pollinator}$ showed similar values between species, being 0.88±0.27 and 0.83±0.31, for *C. filicaulis* and *C. arachnoidea*, respectively (Table 1).

1.3 Post-pollination isolation

Both *Calceolaria* species produced a smaller seed set in interspecific than intraspecific pollinations. However, we could observe some differences when considering pollen reception (Figure 3). *C. filicaulis* conspecific pollination produced three times more seeds (mean ± SE; 453.62 ± 52.11 seed/fruit) than inter-specific crosses when *C. filicaulis* acted as pollen receiver parent (117.75 ± 40.98 seeds/fruit; Wilcoxon signed rank test: W=189, $p < 0.0001$, Figure 3A). *Calceolaria arachnoidea* produced eleven times more seeds in

intraspecific (303.35 ± 50.87 seeds/fruits) than interspecific crosses (25.97 ± 5.10 seeds/fruit; Wilcoxon signed rank test: $W=168, p<0.0001$, Figure 3B). Finally, the strength of the $RI_{post-pollination}$ barrier was different for each plant species (Table 1), being higher for *C. arachnoidea* (0.91 ± 0.04) than for *C. filicaulis* (0.74 ± 0.04).

2. Post-zygotic isolation

2.1 Seed germination

The germination success did not differ between treatments, neither for *C. filicaulis* (Wilcoxon signed rank test: $W=71, p=0.497$, Figure 4A) nor *C. arachnoidea* (Wilcoxon signed rank test: $W=91.5, p=0.716$, Fig. 4B). Mean germination for intraspecific pollination of *C. filicaulis* did not differ from values when *C. filicaulis* was the pollen receiver of *C. arachnoidea* (0.39 ± 0.06 seeds/fruit, and 0.35 ± 0.12 seeds/fruit, respectively). Likewise, mean seed germination for intraspecific pollination of *C. arachnoidea* was 0.27 ± 0.07 seeds/fruit, while interspecific pollination was of 0.23 ± 0.05 seeds/fruit for. The $RI_{viability}$ values were low for both species: 0.11 ± 0.04 for *C. filicaulis*, 0.16 ± 0.04 for *C. arachnoidea* (Table 1).

DISCUSSION

In this study, we estimated the strength of different reproductive barriers in two *Calceolaria* species, in order to determine the factors responsible of the maintenance of species borders in sympatry. As far as we know, our study is the first in documenting the

potential reproductive barriers in *Calceolaria* species.

We estimated three pre-zygotic barriers: two under field conditions (microhabitat and pollination isolation) and one in the greenhouse (intra and interspecific cross-pollination). Seed germination of parental and hybrids were considered as a measure of offspring viability (post-zygotic isolation). In general, species were isolated by geography, the identity of their principal pollinators and by a post-pollination mechanism. Hybrid inviability was not detected in this study, which suggests a minor contribution of post-zygotic barriers to total reproductive isolation in this species.

1. Pre-zygotic isolation

1.1 Microgeographic isolation

Our results showed that *Calceolaria* species are isolated by their spatial distribution within the study site (Table 1). Although pollinator species can fly long distances during the pollination activity, it is possible that microhabitat conditions (e.g., local temperatures, humidity conditions) could be determining the frequency of visits of pollinator species (Totland & Eide 1999), decreasing the inter-specific pollen movement. Even though the importance of geographical isolation in the speciation process is well known (Glennon et al. 2012), micro-geographical isolation has been less investigated (Lowry et al. 2008). The present study places itself between the handfuls of investigations (Ramsey et al. 2003, Kay 2006, Lowry et al. 2011) having quantified the strength of geographical isolation, and is the second one (along with Kay 2006) checking for microgeographical isolation. This study provided two-fold higher $RI_{micro-geographical}$ – values than those reported by Kay (2006) for *Costus* species (20x20 m²: 0.86 ± 0.06 ; 50x50 m²: 0.76 ± 0.06 in our study, versus 0.13 and

0.44 in Kay 2006). It is likely that the two *Calceolaria* species are more patchily distributed in the study site in comparison to *Costus*.

Our study demonstrates that the micro-spatial distribution can affect gene flow between coexisting *Calceolaria* species, stressing the importance of take this variable into account in studies of interspecific reproductive isolation. However, it remains unclear the way this barrier operates during the speciation process as well as the way the local environmental affect pollinator movements (but see Totland 2001).

1.2 Floral visitors

Pollinators contributed to isolation in the two *Calceolaria* species (*C. filicaulis*: 0.88 ± 0.27 ; *C. arachnoidea*: 0.83 ± 0.31). As expected, both plant species were principally visited by different oil-collecting bees (Table 2), which were not observed effecting interspecific pollen exchange (M.Murúa pers. obs.). Nevertheless, both species were also visited by pollen-collecting bees (Table 2). *Calceolaria filicaulis* was the more specialized of the two species, being visited only by two pollinator species, while the pollinator assemblage of *C. arachnoidea* consisted on four different floral visitors (Table 2). Differences in the level of specialization displayed by the two species could be explained by at least two reasons. First, as it has been observed in *Pedicularis* (Yang et al. 2007), differences in floral display may determine the number of pollinators supported per plant (*C. filicaulis*: 2.26 flower/plant; *C. arachnoidea*: 6.58 flower/plant). Second, in the field it was possible to perceive a sweet smell produced by *C. filicaulis* only (pers. obs.), what could be acting as a specific volatile clue for *Chalepogenus*. Although this point might be of high importance in the system, it has been never explored for these species, and future investigations should

study the topic.

Both *Calceolaria* species were mainly visited by distinct oil-collecting bees, suggesting that isolation through pollinators is an important barrier in precluding gene flow. This is also supported by the high *RI* index estimated for the two species (*RI_{filicaulis}*: 0.88 ± 0.27 , *RI_{arachnoidea}*: 0.83 ± 0.31). This discovery is consistent with other studied plant systems, such as *Costus* (Kay 2006), *Ipomopsis* (Aldriege & Campbell 2007), *Mimulus* (Ramsey et al 2003), *Narcissus* (Marqu ez et al. 2007), *Pedicularis* (Yang et al. 2007) and *Penstemon* (Chari & Wilson 2001). Moreover, in a recent review, Lowry et al. (2011) considered 19 cases in which isolation by pollinators had been investigated, concluding that the composition of floral visitors is in most cases an important reproductive barrier. It is important to note that unlike most studies, however, we obtained very high *RI* values (0.83 and 0.88, versus values ranging between 0.1 and 0.6 in other studies). It is likely that unlike this study, most evidence comes from generalized plant-pollination systems. Interestingly, our values are similar to those calculated by Kay (2006) and Ramsey et al. (2003), who also investigated relatively specialized pollination systems. Despite some similarities with those studies, our study represents probably the system with the highest level of specialization (but see Cozzolino & Scopece 2008). In order to determine whether or not pollination specialization truly conveys reproductive isolation, more studies in specialized systems are necessary.

Although the pollination system conformed by *Calceolaria* species and their oil-collecting bees is highly specialized (Molau 1988, S ersic 2004), the plants were also visited by other common non-oil-collecting pollinators, which may help to understand the observation of putative hybrids in the field. Because the only shared pollinator species

observed was the generalist bumblebee *Bombus terrestris* (Table 2). It is likely that this pollen vector contributes to pollen exchange between the two *Calceolaria* species. An obvious shortcoming of this study is the relatively limited sampling effort. Although pollinator visits were recorded during six consecutive days, more hours of observation and measurement of pollen loads are necessary to provide more details on the frequency of cross-visitations and pollen exchange.

1.3. Post-pollination isolation

Pollinators are known to move pollen across long distances, visiting different plant species, and sometimes hampering their reproductive isolation created by other mechanisms. As a response to this, plants have evolved different mechanisms that avoid hybridization, such as, floral phenology, floral mechanical isolation (differences in corolla shape and size) and pollen-style incompatibilities, among others (see reviews in Grant 1994, Costa et al. 2007). In this study, we found strong post-pollination isolation in both directions, albeit the degree of fertilization seemed to depend on the pollen receiver (Figure 3). Specifically, plants doubled their seed set when *C. filicaulis* was the pollen receiver plant, in comparison to crossings where *C. arachnoidea* was the receiver plant (Figure 3). Although relatively high in both cases, the $RI_{\text{post-pollination}}$ index was stronger for *C. arachnoidea* (0.91 ± 0.04) than *C. filicaulis* (0.74 ± 0.04).

To our knowledge, estimates of the strength of post-pollination isolation have been performed only in a limited number of plant species. Compared to such studies, our estimates of post-pollination indexes (*C. arachnoidea*: 0.91; *C. filicaulis*: 0.74) were higher than those estimated by Martin & Willis (2007; *Mimulus*) and Scopece et al. (2013;

Orchis), and similar to those estimated for *Costus* (Kay 2006) and other species of *Mimulus* (Ramsey et al. 2003). Although these results evidence the difficulty to generalize on this topic, differences may relate to variation in the experimental design among studies. Indeed, all the previous studies performed interspecific pollination tests using pollen mixtures of both plant species, making possible that multiples variables (style length, pollen competition or chemistry incompatibility, see below) interfere and reduce hybrid formation (Chari & Wilson 2001). In our study, we used only one pollen donor in all experiments, which may relax interspecific pollen competition leading to an underestimation of the $RI_{post-pollination}$. Even though post-pollination is a widespread isolating barrier, future studies, in artificial and natural conditions, are needed to further identify the ultimate factors responsible of seed development in *Calceolaria* species.

As mentioned above, the asymmetry of interspecific cross-pollination observed in our study system has been also documented for other plants species. In general, two mechanisms have been invoked for such pattern: style length and pollen competition, and at least one of them could be applied to our case (Arnold 1997). First, in our case, differences in style length between the two species seem to be the most parsimonious explanation for the reduction of the reproductive success in the interspecific treatment. When interspecific differences in style length exist, the species with shorter styles can be more easily fecundated than the species with longer styles (e.g., Chari & Wilson 2001, see Tiffin et al. 2001), since the ovary will be more easily reached in the first than in the second case (Howard 1999). Here, the style length of *C. arachnoidea* is longer than that of *C. filicaulis* (mean \pm SE; *C. filicaulis*: 1.62 ± 0.04 cm.; *C. arachnoidea*: 2.43 ± 0.05 cm), suggesting that the rule of the style length applies in this case. Second, pollen competition has been

also documented as another strong post-pollination barrier (e.g., Campbell et al. 2003, Ramsey et al. 2003). When conspecific and heterospecific pollen are competing in the same style, heterospecific pollen tubes may grow at a slower rate or show a greater attrition than conspecific ones, with the degree of isolation being dependent upon the stylar tissue (Chari & Wilson 2001). Examples of pollen competition have been documented for different species, such as *Chamerion* (Husband et al. 2002), *Louisiana* (Emms et al. 1996), and *Mimulus* (Diaz & Macnair 1999). Even though this barrier may be also present in our species, we do not have the required information at present to evaluate such possibility. Indeed, the interspecific cross-pollination experiment was performed only with pollen of one plant species at the time, not with pollen mixture. In order to investigate this point, future studies should also try to evaluate this issue.

2. Post-zygotic isolation

2.1 Seed germination

Our results revealed a lack of post-zygotic barrier to gene flow between the two studied *Calceolaria* species. Indeed, hybrids performance in terms of seed germination did not differ from parental plants. Although the viability isolation index was lower in *C. filicaulis* (0.11 ± 0.04) than *C. arachnoidea* (0.16 ± 0.04), both species showed that hybrid sterility is a weak barrier to reproductive isolation. These results contrast with those observed in *Costus* (Kay 2006), *Orchis* (Scopece et al 2013) and *Pitcairnia* (Wendt et al. 2001), where hybrids had a low germination and parental plants had high germination. It is likely that difference between our results and those from previous studies base on the small genetic distance separating the two *Calceolaria* species (Winder et al. 2009). It is important to note that

despite hybrid seeds germinated at the same proportion as parental plants, the effect on long-term survival rate and fertility is unknown, as successive stages of the life cycle were not evaluated. It is likely that other factors not considered in this study influence plant long-term performance and reproductive isolation (see Reiseberg & Willis 2007). These rates may be influenced by intrinsic developmental barriers, such as potential pollen sterility (Ohta 1999) or extrinsic ones occurring at the F₂ and/or in backcrossed generations, through pollinator avoidance (Tastard et al. 2008, Andalo et al. 2010) and as a consequence of the phenotypic variation related to hybridization. Another extrinsic factor affecting post-zygotic survival could be related to local abiotic environmental conditions, which could be selecting against hybrid seeds. It is important to note that germination success was the only post-zygotic isolation barrier evaluated in this study, and other non-considered post-zygotic barriers (e.g., hybrid fertility, hybrid breakdown) could be also playing a major role in the reproductive isolation of these species.

More work will be necessary to determine if the isolation pattern reported here is a global pattern of the Chilean *Calceolaria* species or is local dependent phenomenon. Consequently, futures studies incorporating other reproductive barriers (i.e., intrinsic and extrinsic), as well as other localities, will be required in order to arrive this goal.

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TABLES

Table 1. Indexes of reproductive isolation for the two studied sympatric *Calceolaria* species. Reproductive isolation indexes (*RI*) are showed for each species.

Isolating barriers	Strength of reproductive isolation (<i>RI</i>)	
	<i>C. filicaulis</i>	<i>C. arachnoidea</i>
Micro-geographical	0.76±0.06	0.76±0.06
Pollinator	0.88±0.27	0.83±0.31
Post-pollination	0.74±0.04	0.91±0.04
Seed germination	0.11±0.04	0.16±0.04

Table 2. Floral visitors of the two studied sympatric *Calceolaria* species. Identity of pollinators, mean visitation rate (\pm SE), and proportion of visits per insect species.

Plant Species	Pollinators	Visitation rate [Visits/(flower*h)]	Proportion of visits
<i>C. filicaulis</i>	<i>Chalepogenus caeruleus</i>	0.29 \pm 0.07	0.831
	<i>Bombus terrestris</i>	0.06 \pm 0.03	0.169
<i>C. arachnoidea</i>	<i>Centris nigerrima</i>	0.30 \pm 0.04	0.872
	<i>Bombus terrestris</i>	0.04 \pm 0.20	0.121
	<i>Chalepogenus</i> sp. 1	0.002 \pm 0.002	0.006
	<i>Megachile semirufa</i>	0.001 \pm 0.001	0.001

FIGURES

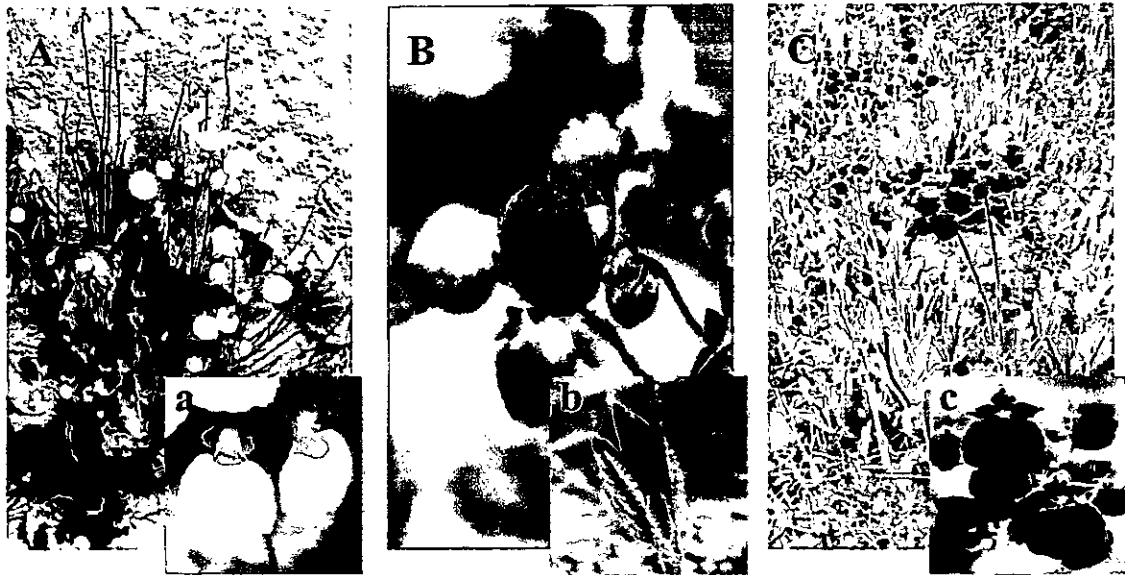


Figure 1. *Calceolaria* species and the putative hybrids between both species founded in National Reserve Altos de Lircay. A) *C. filicaulis* (a: corolla), B) Hybrids (b: leaf), C) *C. arachnoidea* (c: corolla).

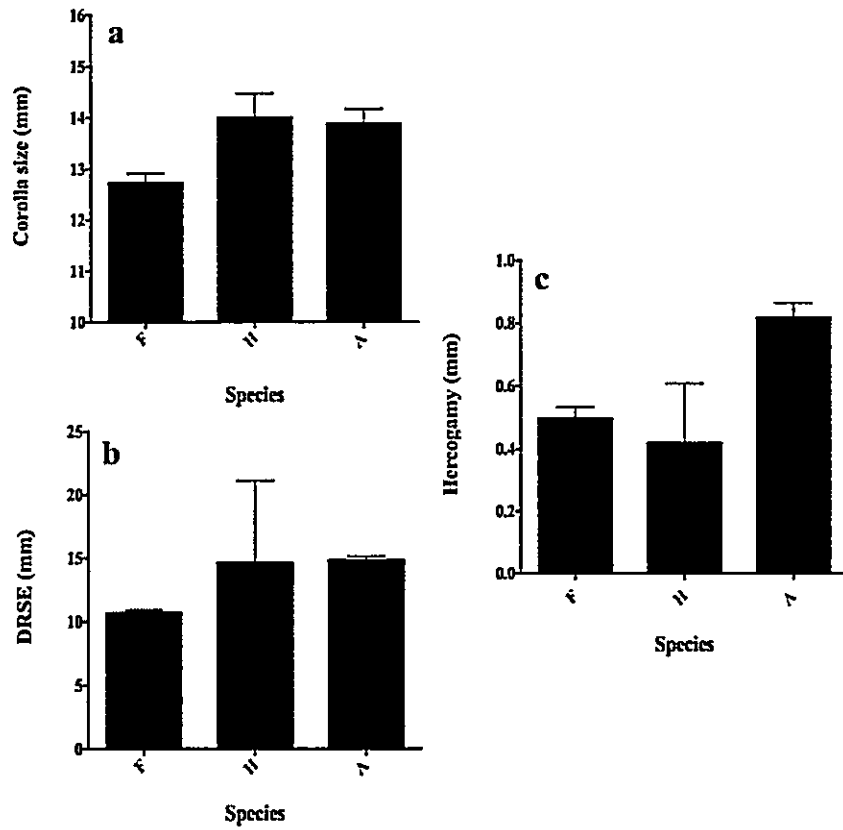


Figure 2. Corolla traits of *Calceolaria* species and their putative hybrids. A) *C. filicaulis* (F), Hybrid (H), and *C. arachnoidea* (A). Corolla traits: a) Corolla size, b) Distance between reproductive structures and the elaiophore (DRSE) and c) Hercogamy. Bars and error bars represent means and standard errors, respectively.

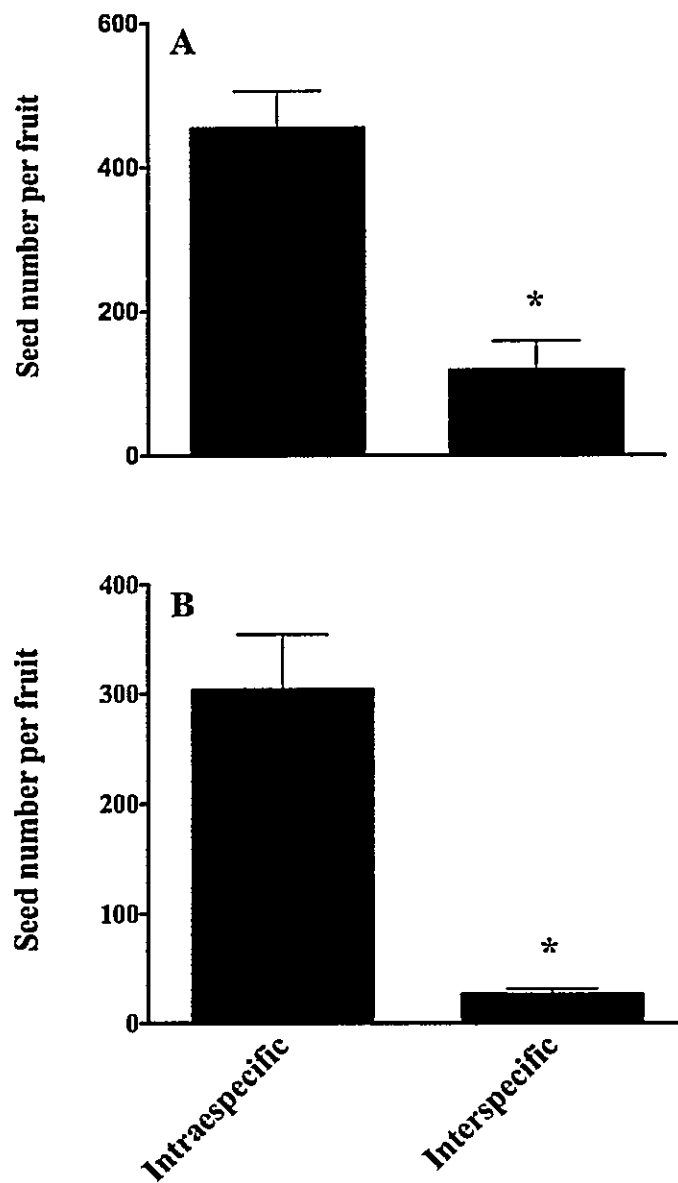


Figure 3. Seed production (seed number per fruit) by intra- and interspecific pollination crosses of (A) *C. filicaulis* and (B) *C. arachnoidea*. Asterisks over the bars depict statistical effects at $P < 0.001$. Bars and error bars represent means and standard errors, respectively.

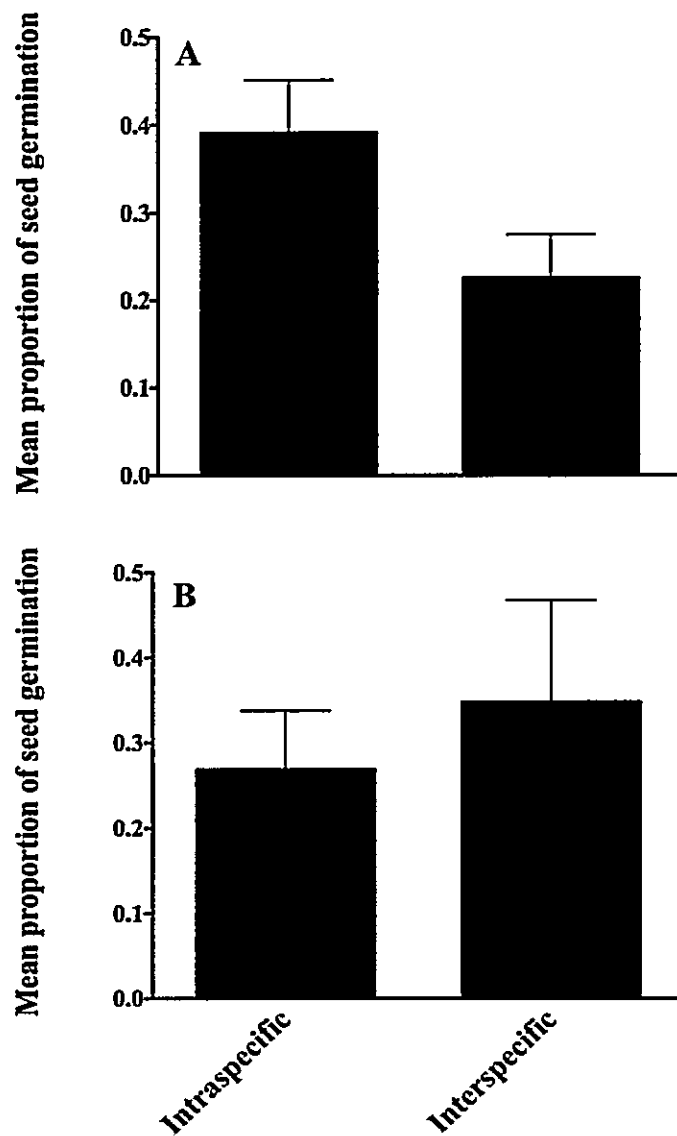


Figure 4. Proportion of germinated seed produced by intra- and interspecific pollination crosses for (A) *C. filicaulis* and (B) *C. arachnoidea*. Asterisks over the bars depict statistical effects at $P < 0.001$. Bars and error bars represent means and standard errors, respectively.

CAPITULO V

CONCLUSIONES

En las últimas tres décadas, el creciente número de estudios en método comparado ha proporcionado a la biología evolutiva una valiosa herramienta para la detección del posible rol de los polinizadores en la evolución floral (Smith 2010). Si bien esta herramienta resulta complementaria a otras aproximaciones como estudios micro-evolutivos de selección mediada por polinizadores (ver revisión en Trip & Manos 2008), esta permite obtener patrones generales que sirven como punto de partida para estudiar en detalle los procesos y mecanismos evolutivos que los explican (van der Niet & Johnson 2012). En los últimos años, diversos estudios comparativos en distintos linajes de plantas han corroborado el rol de los polinizadores como principales agentes de cambio evolutivo (e.g., Kay et al. 2005, Pérez et al. 2006, Smith et al. 2008). Gran parte de estas investigaciones han sido llevadas a cabo en sistemas generalistas, donde estudios en sistemas especialistas son escasos. Sin embargo, estos últimos representan modelos únicos para explorar los factores que moldean la evolución de las interacciones planta-animal.

De acuerdo a lo anterior, el sistema especialista conformado por *Calceolaria* y sus abejas recolectoras de aceites, podría representar un buen sistema para evaluar el rol de los polinizadores en la evolución de los sistemas de polinización. En este sentido, el presente trabajo de tesis tuvo como primer objetivo determinar si la evolución de los caracteres florales de nueve especies de *Calceolaria* estuvo determinada por las relaciones ancestro-

descendiente o respondía más bien a contingencias ecológicas. Consecuentemente, si la historia evolutiva no presenta ningún efecto sobre la evolución de rasgos en *Calceolaria* se probó la hipótesis de que la morfología floral presentaría una correspondencia con la morfología de sus principales polinizadores. En general, nuestros resultados mostraron ausencia de señal filogenética para cuatro de los cinco rasgos florales, patrón que ha sido descrito con anterioridad para diversas especies de plantas tales como, *Gladiolus* (Valente et al. 2012), *Disa* (Johnson et al. 1998), *Aquilegia* (Whittall & Hodges 2007) o *Costus* (Kay et al. 2005), lo que respaldaría la naturaleza lábil de los rasgos florales en las Angiospermas. Por otra parte, de los cinco rasgos estudiados solo el área de la corola presentó un mínimo efecto de la filogenia. Lo anterior no resulta sorprendente, ya que investigaciones recientes han obtenido resultados similares para otras especies de plantas, sugiriendo que rasgos asociados al tamaño de la corola evolucionarían de manera más conservativa que otros caracteres florales (e.g., estructuras reproductivos) en respuesta a un desarrollo común (Conner & Sterling 1995, Diggle 2002, Alcantara & Lohmann 2011), o desde una mirada adaptativa, a la atracción de un grupo particular de polinizadores (e.g., polinizadores especialistas; Alcantara & Lohmann 2011). Entonces, la ausencia de señal filogenética sugeriría que los rasgos florales en *Calceolaria* podrían haber evolucionado principalmente en respuesta a la contingencia ecológica (e.g., polinizadores) más que a una relación ancestro-descendiente. Desafortunadamente, la ausencia de correlación entre los rasgos florales y la morfología de los polinizadores no permitió determinar si los polinizadores son la principal fuerza evolutiva afectando la diversificación floral en *Calceolaria*. No obstante, este hecho no puede ser descartado dado que el sistema planta-polinizador entre *Calceolaria* y sus abejas colectoras de aceites ha sido descrito como una

relación que ha evolucionado recientemente (6-1Myr; Renner & Schaefer 2010), por lo que es posible que la interacción entre ellos se encuentre aún en proceso de ajuste morfológico.

Por otra parte, y dado que la relación entre adaptación y especiación continúa siendo un tema central en biología evolutiva, en particular en sistemas sobre los cuales se tiene poco conocimiento, es que el segundo objetivo de esta investigación comprendió determinar las barreras al flujo génico entre dos especies de *Calceolaria*. Específicamente se exploraron diferentes barreras al aislamiento reproductivo (pre y post-cigóticas) entre *C. filicaulis* y *C. arachnoidea*, que habitaban en simpatría en la región del Maule. Adicionalmente, se quiso explorar la hipótesis de que los polinizadores son los principales responsables de impedir el flujo génico entre las especies, dada la alta especialización de este sistema de estudio. Globalmente, los resultados corroboraron a los polinizadores como uno de los principales responsables de la mantención de la identidad de estas especies en simpatría. En general, se observó que las especies de *Calceolaria* estuvieron aisladas tanto por su posición geográfica en el sitio de estudio, como por la identidad de sus principales polinizadores, y la incompatibilidad de polen inter-específico entre ellas. Si bien todas las barreras pre-cigóticas antes mencionadas contribuyeron fuertemente al aislamiento de estas especies en distintas etapas de su ciclo de vida, es indudable que los polinizadores juegan un rol principal en el limitado flujo génico existente entre las especies estudiadas. Esta idea adquiere relevancia si se considera que cada especie de *Calceolaria* fue polinizada exclusivamente por un grupo de abejas recolectoras de aceite distinto (i.e., *Chalepogenus* visitó a *C. filicaulis*, mientras que *Centris* visitó a *C. arachnoidea*). Si bien es posible que los polinizadores no puedan impedir completamente el flujo genético entre las plantas, es sabido que éstos representan una de las principales -si no la principal- barreras de

aislamiento reproductivo y de diversificación en muchos otros grupos de plantas (ver Johnson et al. 1998, Alcantara & Lohmann 2010, Lowry et al. 2011). Estas ideas toman mayor fuerza específicamente en el caso del género *Calceolaria*, para el cual se ha sugerido una evolución y diversificación simultánea al establecimiento de la interacción de recolección de aceites (Molau 1988, Sersic 2004, Cosacov et al. 2009) en el Terciario tardío (6-1 Myr; Renner & Schaefer 2010).

Los resultados de este trabajo muestran la importancia de la integración de los aspectos filogenéticos y ecológicos en los estudios de evolución de sistemas de polinización. Esto es particularmente importante en sistemas de polinización especialistas, ya que por su baja frecuencia han sido menos estudiados. En ese sentido, lo reportado por esta investigación resulta novedoso y pionero en el estudio de las relaciones evolutivas entre las especies de *Calceolaria* y sus polinizadores.

Bajo este escenario, surge la necesidad de seguir avanzando en el conocimiento tanto básico como aplicado de uno de los sistemas más especializados que han sido descritos recientemente en la literatura (Cosacov et al. 2009). Con este fin, futuros estudios deberán tratar de identificar los factores últimos que han moldeado la evolución de estas complejas estructuras florales, así como también la relación recíproca entre las mismas y sus polinizadores.

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