UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

ESCUELA NACIONAL DE ESTUDIOS SUPERIORES, UNIDAD MORELIA

Evolución de los caracteres de atracción e integración floral del género *Achimenes* (Gesneriaceae).

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

ERANDI RAMÍREZ AGUIRRE

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MORELIA, MICHOACÁN, AGOSTO, 2020.

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OFICIO CPCB/470/2020

ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
Presente

Me permito informar a usted que en la reunión ordinaria del Subcomité de Biología Evolutiva, Ecología, Manejo Integral de Ecosistemas y Sistemática del Posgrado en Ciencias Biológicas, celebrada el día **20 de enero de 2020** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **RAMÍREZ AGUIRRE ERANDI** con número de cuenta **515025906** con la tesis titulada **"Evolución de los caracteres de atracción y de la integración floral en el género Achimenes (Gesneriaceae)"**, realizada bajo la dirección de la **DRA. SILVANA MARTÉN RODRÍGUEZ**, quedando integrado de la siguiente manera:

Presidente:	DR. ALFONSO DELGADO SALINAS
Vocal:	DR. JUAN ENRIQUE FORNONI AGNELLI
Secretario:	DR. MAURICIO RICARDO QUESADA AVENDAÑO
Suplente:	DR. ANTONIO GONZÁLEZ RODRÍGUEZ
Suplente:	DRA. MARIA DEL CORO ARIZMENDI ARRIAGA

Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd. Mx., a 10 de agosto de 2020

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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A mi familia.

TABLA DE CONTENIDO

RESUMEN

La diversificación de las angiospermas se ha asociado a los cambios en el sistema de polinización y estrategias reproductivas de las plantas. Existe evidencia de convergencia evolutiva en caracteres florales como el color, el néctar o las fragancias, asociada a cambios en los polinizadores principales de las plantas. Las diferentes asociaciones de caracteres florales conforman los denominados síndromes de polinización. A nivel de especie, los caracteres florales pueden estar correlacionados entre sí (e.g. largo y ancho de la corola, largo de estambres y pistilos), lo que sugiere que puede haber selección correlacional mediada por los polinizadores más frecuentes y eficientes. En esta tesis se evaluó la evolución de caracteres florales y de integración floral en el género *Achimenes* (Gesneriaceae), que está compuesto por alrededor de 25 especies con morfologías y colores diferentes. Aproximadamente el 40% de las especies se encuentra en simpatría pues se encuentran en zonas rocosas escarpadas de alta humedad. Esto sugiere que la diversificación floral en el género *Achimenes* podría ser también resultado de selección mediada por polinizadores para promover el aislamiento reproductivo. En esta tesis se han propuesto las siguientes preguntas: (1) ¿cómo se asocian las características florales a los polinizadores de las diferentes especies de la familia Gesneriaceae?, (2) ¿qué barreras reproductivas permiten la coexistencia de especies del género *Achimenes* en simpatría?, (3) en un contexto filogenético, ¿están asociados los cambios en las características florales como el color, tamaño y forma de la corola a los cambios de polinizador en el género *Achimenes*?, (4) ¿cómo varían los patrones de integración de las características florales entre síndromes?

Encontré que los cambios de estados en el color, el tamaño y la forma de la coinciden con los cambios de sistema de polinización observados y que el valor de integración de la corola es similar entre especies, aunque tengan patrones de covarianza diferentes.

ABSTRACT

Angiosperm diversification has been associated with canches in pollination sistems and to other reproductive strategies of plants. There is evidence that convergence of floral characters, such as color, nectar or fragrances, is associated with changes of plant principal pollinators. The different associations of floral characters make up the so-called pollination syndromes. At the species level, the floral characters may be correlated with each other (e.g. length and width of the corolla, length of stamens and pistils), suggesting that there may be correlational selection mediated by the most frequent and efficient pollinators. In this thesis, I evaluated the evolution of floral characters and floral integration in the *Achimenes* genus (Gesneriaceae), which is represented by around 25 species with different morphologies and colors. Approximately 40% of the species are in sympatry, as they are found in steep rocky areas with high humidity. This suggests that floral diversification in the *Achimenes* genus could also be the result of pollinator-mediated selection to promote reproductive isolation. The following questions have been proposed in this thesis: (1) how are the floral characteristics associated with pollinators of the different species of the Gesneriaceae family?, (2) what reproductive barriers allow the coexistence of species of the genus *Achimenes* in sympatry?, (3) within a phylogenetic context, are changes in floral characteristics such as color, size and shape of the corolla associated with pollinator changes in the *Achimenes* genus ?, (4) how do integration patterns of the floral characteristics vary between syndromes? I found that the changes in color, size and shape of the states coincide with the observed pollination system changes and that the integration value of the corolla is similar between species, although they have different covariance patterns.

INTRODUCCIÓN GENERAL

La diversidad floral de las angiospermas ha resultado en parte de procesos de adaptación derivados de la interacción con polinizadores particulares que favorecen la transferencia de polen de una planta a otra (van der Niet & Johnson 2012). La diversidad floral es evidente en caracteres como: largo de la corola, forma de la flor, tamaño de la flor, la producción de recompensas, como el néctar o las fragancias, y el color de la corola (Fenster *et al.*, 2004; Rosas-Guerrero *et al.*, 2014; Schiestl & Johnson 2013). Dichos caracteres están asociados con las capacidades sensoriales, como la visión en el espectro ultravioleta o la detección de fragancias, y las necesidades nutricionales de los polinizadores (e.g. Fleming *et al*., 2004; Papiorek *et al.*, 2016; Raguso *et al.*, 2004). Stebbins propuso que la frecuencia en las visitas y la eficiencia en la transferencia de polen son claves para que un polinizador particular genere una presión selectiva en la morfología floral (1970). Esto indicaría que hay polinizadores principales que se espera sean los principales agentes de selección sobre las características florales.

Derivado de las presiones selectivas de polinizadores particulares y su asociación con caracteres florales, se han conceptualizado los síndromes de polinización. Estos se definen como la agrupación de caracteres florales que pueden estar correlacionados entre sí, y que sirven para atraer ciertos tipos de polinizadores y fomentar la transferencia efectiva de polen (Fenster *et al.*, 2004). La existencia de los síndromes se ha corroborado en diversas especies de diferentes familias de angiospermas, lo que implica convergencia en las presiones selectivas por parte de los polinizadores. Por ejemplo, las flores polinizadas por colibríes de numerosas familias de plantas -e.g. Gesneriáceas, Convolvuláceas o Heliconiáceaspresentan corolas de colores brillantes, rojas y tubulares, con cantidades abundantes de néctar diluido y con antesis diurna (Proctor *et al.*, 1996). Esto sugiere que los polinizadores no sólo ejercen presiones de selección sobre los caracteres florales particulares, sino que pueden promover cambios entre síndromes de polinización (Ashworth *et al.*, 2015). Sin embargo, se conoce poco acerca de cómo evolucionan los conjuntos de caracteres florales cuando hay un cambio de un polinizador a otro y si los cambios florales ocurren en conjuntos de caracteres o independientemente entre sí.

Entre los modelos que se han usado para explicar los cambios florales generados por cambios de polinizador se ha propuesto que es la disyuntiva entre la ganancia en adecuación asociada a un polinizador más eficiente y la ganancia en adecuación asociada con un polinizador menos eficiente (Aigner, 2001; Muchhala, 2007). Por ejemplo, el cambio de polinización por murciélago a colibrí implica cambios en el ancho de la corola, que afectan a su vez la eficiencia en la transferencia de polen (Muchhala, 2007). En otros modelos, la diferencia en la adecuación es afectada por la abundancia de un tipo de polinizador con respecto a otro, lo que favorece más los cambios hacia sistemas especializados y mixtos (Sargent & Otto, 2006). El reforzamiento, i.e. la selección sobre ciertas características fenotípicas que promueven el aislamiento reproductivo, también podría favorecer el cambio de un sistema de polinización a otro, en el que las especies hermanas tenderían a tener sistemas de polinización convergentes al encontrarse en simpatría (Bank *et al.*, 2012; Hopkins, 2013; Kay & Schemske 2008). Aún si alguna de las hipótesis está más frecuentemente apoyada por datos empíricos, todas son hipótesis adaptativas en las que los polinizadores son agentes selectivos que median los cambios en las características florales. A nivel filogenético, dichas presiones selectivas se verían reflejadas en los patrones de convergencia de los síndromes de polinización en diferentes linajes de especies (Losos, 2011). Además, no toda la variación en características florales es producto de procesos de adaptación a polinizadores, ya que también hay otras fuerzas evolutivas responsables de algunos patrones de diversidad floral, como los procesos de desarrollo, genéticos e interacciones con otros organismos (Strauss & Whittall, 2006; Wessinger & Hileman, 2016; Smith, 2016).

En general, los estudios comparativos que han evaluado la evolución tanto de las características florales como de los sistemas de polinización, sugieren que los cambios en el color o la forma de la corola concuerdan con los cambios en el sistema de polinización (e.g. Forest *et al.*, 2014; Martén-Rodríguez *et al.*, 2010; Tripp & Manos, 2008; Whittall & Hodges, 2007; Wilson *et al.*, 2007). Sin embargo, la mayoría de los estudios publicados se basan en las características del síndrome floral para inferir los polinizadores o tienen un muestreo incompleto de los polinizadores (menos del 50% de las especies del taxón de estudio). Aunque se ha demostrado que los síndromes de polinización son capaces de predecir los polinizadores más eficientes en muchas especies de plantas, existen especies cuyos polinizadores primarios no corresponden a los predichos por el síndrome o cuyo sistema de polinización comprende más de un grupo de polinizadores (Rosas-Guerrero *et al.*, 2014). Además, pocos trabajos consideran la evolución de caracteres cuantitativos como parte de los fenotipos florales, aunque éstos pudieran dar indicios de cómo cambian las características florales en conjunto (i.e. correlaciones entre caracteres florales) (e.g.Smith, 2010; Landis *et al.*, 2018; Benitez-Vieyra *et al.*, 2019).

Las flores son fenotipos complejos e integrados por diferentes caracteres (como el largo de las anteras o el pistilo, largo de la corola, ancho de la corola, etc.) que covarían entre sí y pueden representar procesos de desarrollo, genéticos y de funcionamiento concertado

(Armbruster *et al.*, 2014). Dado que muchas especies dependen de animales para la transferencia de polen, se ha propuesto que las flores de especies que requieren polinizadores para su reproducción tendrían correlaciones más altas entre caracteres florales que las que no dependan de animales para su reproducción (Berg, 1960). Berg (1959) también propuso que habría correlaciones más altas entre los caracteres directamente asociados con la transferencia de polen, como morfología de las anteras y los estigmas, en comparación con la correlación entre partes reproductivas con partes no involucradas en la polinización, como las hojas. Las ideas de Berg, representan una hipótesis adaptativa, en la que el agente de selección que genera las correlaciones morfológicas florales es el polinizador. Existen algunos trabajos a nivel poblacional que han puesto a prueba las ideas de Berg y que las apoyan (e.g. Lázaro & Santamaría, 2015; Pérez-Barrales *et al.*, 2007; Reynolds *et al.*, 2010), aunque también existen estudios que indican que las presiones selectivas de los polinizadores no favorecen una mayor integración entre las partes florales, sino que son los procesos de desarrollo los que favorecen la mayor integración (e.g. Herrera *et al.*, 2002). En el caso de la biología comparativa, ocho estudios han evaluado la evolución de la integración floral mediante la reconstrucción de la integración floral ancestral en la filogenia, mapeando las correlaciones entre caracteres florales medidos o mediante regresiones en las que los polinizadores son predictores de los caracteres florales y de su covariación (por el método de cuadrados mínimos generalizados filogenéticos) (Pérez *et al.*, 2007; Rosas-Guerrero *et al.*, 2010; Alcántara *et al.*, 2013; Gómez *et al.*, 2014; Gómez *et al.*, 2016; Smith & Kriebel, 2018; Benitez-Vieyra *et al.*, 2019; Reich *et al.*, 2020). En estos trabajos, los resultados sugieren que las flores de las especies autocompatibles (que dependen menos de los polinizadores) están más integradas que las autoincompatibles, así mismo, las especies con sistemas de polinización más especializados (i.e. con menor diversidad de polinizadores) tienen flores más integradas que las flores más generalistas. Para el estudio de la diversificación y adaptación de las angiospermas a los polinizadores es necesario aumentar el muestreo de especies dentro del taxón y que tengan representantes de diferentes síndromes de polinización. Además, idealmente cualquier trabajo que analice la evolución de los síndromes de polinización y evolución floral debería incluir observaciones de polinizadores en campo y mediciones más detalladas de la morfología y recompensas florales para construir hipótesis adaptativas más sólidas. Mientras que, para conocer los patrones de cambios entre síndromes de polinización en un contexto comparativo, idealmente se deben estudiar grupos de especies cercanamente emparentadas, que presenten una alta diversidad floral y potencialmente más de un polinizador.

Los polinizadores también pueden favorecer la diversificación floral a través del aislamiento reproductivo, al mantener la integridad genética de las especies de angiospermas (Baack *et al.,* 2015). Aparte de la importancia que tienen los polinizadores en la diversificación a nivel macroevolutivo, los polinizadores pueden jugar un papel importante en los procesos de divergencia poblacional y especiación, ya que pueden promover o reducir el aislamiento reproductivo entre especies cercanamente emparentadas. Cuando las especies emparentadas coexisten y se cruzan entre sí, la adecuación e integridad genética de las especies progenitoras puede ser afectada negativamente. Por ejemplo, los gametos que podrían ser utilizados en la reproducción intraespecífica serían desperdiciados cuando el polen es depositado sobre estigmas interespecíficos, cuando los estigmas son bloqueados con polen interespecífico, o cuando se asignan recursos hacia la formación de embriones que posteriormente serán abortados (Moreira-Hernández & Muchhala, 2019). La descendencia híbrida también representa un costo si no es viable o es poco competitiva en comparación con la descendencia intraespecífica, porque la selección actúa en contra de los híbridos o del proceso de hibridación a través de la evolución de los mecanismos de aislamiento reproductivo (Servedio & Noor, 2003).

Entre los mecanismos que pueden limitar el flujo genético entre las especies de angiospermas, están el aislamiento ecogeográfico, la diferenciación en la fenología floral, el aislamiento asociado a la morfología de los polinizadores y su conducta (mecánico y etológico), todos como mecanismos precigóticos (Grant, 1994; Sobel *et al.*, 2010). Existen también los mecanismos postcigóticos de aislamiento reproductivo, los cuales actúan sobre la descendencia híbrida, por ejemplo, la baja viabilidad de los híbridos, la baja fertilidad híbrida y la baja capacidad para competir en el ambiente natural con las especies parentales (Orr & Turelli, 2001; Coyne & Orr, 2004). Aunque la importancia de cada mecanismo de aislamiento reproductivo varía dependiendo de la especie y está ponderada con respecto a otros mecanismos de aislamiento (Lowry *et al.*, 2008; Sobel & Chen, 2014), las barreras de aislamiento reproductivo actúan en conjunto, permitiendo o facilitando la divergencia entre linajes (Kay & Sargent, 2009). Es decir, el aislamiento reproductivo es el contexto en el que los cambios tanto genéticos como fenotípicos pueden generar nuevas especies (Rieseberg & Willis, 2007).

Para entender la importancia de los mecanismos de aislamiento reproductivo en la diversificación y mantenimiento de las especies, es necesario estudiar poblaciones de especies hermanas o cercanamente emparentadas en situaciones simpátricas e idealmente, también en poblaciones alopátricas. En particular, para la evaluación de los mecanismos postcigóticos derivados de cruzas interespecíficas, es preferible que las plantas estudiadas tengan períodos de vida cortos y manejables para poder realizar cruzas interespecíficas y hacer seguimientos de la descendencia híbrida dentro de invernaderos.

Para este trabajo se propuso al género *Achimenes* (Gesneriaceae) que comprende entre 24 y 26 especies, de las cuales, todas se encuentran en México, aunque existen poblaciones de diversas especies en Centroamérica y hasta el norte de Sur América (Ramírez-Roa, 1987). Las especies de *Achimenes* habitan en microambientes con alta humedad, generalmente sombreados, sobre rocas y pendientes, muchas veces en hábitats estacionales. La mayoría de las especies de encuentra asociada a cuencas hidrológicas, aunque pueden ser también abundantes en paredes rocosas húmedas y cavernas. Las flores de las especies de este género presentan variación en la coloración de la corola, el largo y forma de la corola y el tamaño de la flor. Además, son hierbas pequeñas que pueden ser mantenidas en invernadero, y se han cultivado en otros países de Norte América desde hace más de 100 años. Una particularidad del género *Achimenes* que comparte con otros géneros de Gesneriaceae, es que las especies son relativamente fáciles de hibridar, incluso de forma intergenérica. Sin embargo, los híbridos no parecen ser abundantes o comunes en los ambientes naturales. Esto sugiere que hay una alta especialización por parte de los polinizadores como abejas, colibríes, y mariposas, que no son compartidos entre sí. Otra posibilidad, no excluyente, es que la progenie derivada de cruzas tanto interespecíficas como intergenéricas, sea poco competitiva o poco atractiva para los polinizadores, lo que reduce su adecuación en la naturaleza.

En la presente tesis, se presentarán tres capítulos. El primer capítulo es un artículo publicado (Ramírez-Aguirre *et al.*, 2019) en el que se evaluaron los mecanismos de aislamiento reproductivo pre y post cigóticos entre tres especies simpátricas de *Achimenes*, en el estado de Michoacán, México. El segundo capítulo trata de la evolución de la integración floral, del color, forma y morfología floral y su relación con los polinizadores observados en 21 especies del género *Achimenes*. El tercer capítulo es una revisión de los diferentes aspectos de la biología reproductiva de la familia Gesneriaceae, en la que abordamos la relación de los polinizadores con las características florales y su influencia en la diversidad floral en dicha familia. En esta tesis se muestra cómo las presiones de selección que ejercen los polinizadores pueden favorecer tanto la divergencia como la convergencia de caracteres florales dentro de un género de angiospermas.

CAPÍTULO I. REPRODUCTIVE ISOLATION AMONG THREE SYMPATRIC *ACHIMENES* SPECIES: PRE- AND POST-POLLINATION COMPONENTS.

RESUMEN

PREMISA: Las especies estrechamente relacionadas que se producen en simpatía pueden experimentar efectos negativos consecuencia de la transferencia de polen interespecífica si las barreras de aislamiento reproductivo (RI) no se encuentran. Evaluamos la importancia de las barreras RI, tanto pre como postpolinización en tres especies simpáticas de *Achimenes* (Gesneriaceae), las barreras evaluadas fueron, las ecogeográficas, fenológicas, el aislamiento floral, la autopolinización y la viabilidad híbrida (producción de frutos y semillas).

MÉTODOS: Registramos los rangos de distribución geográfica de las especies y evaluamos la fenología de floración y visitas a polinizadores en un sitio en el centro de México. En invernadero, medimos los caracteres florales involucrados en RI y cuantificamos el conjunto de frutas y semillas para de cruces propios, intraespecíficos e interespecíficos.

RESULTADOS: Las barreras ecogeográficas fueron importantes en RI, pero, en simpatría, la fenología y el aislamiento florales contribuyeron más al IR total. El IR fenológico varió entre especies y años, mientras que el RI floral fue 100% efectivo para prevenir las visitas interespecíficas. Las especies mostraron diferencias en la morfología floral, el color y las fragancias asociadas con diferentes sistemas de polinización (*A. antirrhina*-colibríes, *A. flava*-abejas, *A. patens*-mariposas); los eventos de visita heteroespecífica se restringieron a polinizadores secundarios raros. Las cruzas híbridas produjeron consistentemente menor número de progenie que los cruces intraespecíficos.

CONCLUSIONES: Este estudio indica que ni la autogamia ni la post polinización temprana impiden el flujo de polen interespecífico entre las especies de *Achimenes*. Sin embargo, el aislamiento floral, que actúa a través de la combinación de caracteres de atracción y recompensa, consistentemente garantiza la especificidad de los sistemas de polinización. Estos resultados sugieren que la selección de caracteres florales para reducir los costos de la producción de progenie híbrida podrían haber jugado un papel en la evolución o mantenimiento de sistemas de polinización especializados en *Achimenes*.

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RESEARCH ARTICLE

Reproductive isolation among three sympatric *Achimenes* species: pre- and post-pollination components

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PREMISE: Closely related species occurring in sympatry may experience the negative consequences of interspecific pollen transfer if reproductive isolation (RI) barriers are not in place. We evaluated the importance of pre- and post-pollination RI barriers in three sympatric species of *Achimenes* (Gesneriaceae), including ecogeographic, phenological, floral isolation, self-pollination, and hybrid viability (fruit and seed set).

METHODS: We recorded geographic distribution throughout species ranges and assessed flowering phenology and pollinator visitation at one site in central Mexico. In the greenhouse, we measured floral traits involved in RI and quantified fruit and seed set for from self, intraspecific, and interspecific crosses.

RESULTS: Ecogeographic barriers were important in RI, but under sympatry, phenological and floral barriers contributed more to total RI. Phenological RI varied between species and years, while floral RI was 100% effective at preventing interspecific visitation. Species showed differences in floral morphology, color, and scents associated with specialized pollination systems (*A. antirrhina*–hummingbirds, *A. flava*–bees, *A. patens*–butterflies); heterospecific visitation events were restricted to rare secondary pollinators. Hybrid crosses consistently yielded progeny in lower numbers than intraspecific crosses.

CONCLUSIONS: This study indicated that neither autogamy nor early post-pollination barriers prevent interspecific pollen flow between *Achimenes* species. However, floral isolation, acting through a combination of attraction and reward traits, consistently ensures specificity of the pollination system. These results suggest that selection on floral traits to reduce the costs of hybrid progeny production may have played a role in evolution or maintenance of specialized pollination systems in *Achimenes*.

KEY WORDS floral isolation; Gesneriaceae; hybridization; pollination; reproductive interference; sympatry; temporal isolation.

The co-occurrence of closely related plant species in the same community poses a challenge for species that share similar habitats or resources. At sympatric sites, congeneric species may not only compete for abiotic resources and pollinators, but they are also vulnerable to reproductive interference (i.e., interspecific reproduction that reduces the fitness of at least one of the coexisting species; Kyogoku, 2015). The phenotypic traits that prevent reproductive interference may evolve in allopatry or sympatry, but in coexisting populations of congeneric species, they may act to prevent interspecific gene flow and gamete wastage and the concomitant costs of hybrid progeny production (Hopkins and Rausher, 2012). When interspecific pollination represents a cost to reproduction, mechanisms of reproductive isolation (RI) allow congeneric species to coexist with reduced reproductive interference (Coyne and Orr, 2004).

Reproductive isolation barriers generally evolve during speciation or upon secondary contact between recently diverged species. In plants, these are divided into pre- and post-pollination mechanisms according to their timing of action within the reproductive cycle of the plant (Baack et al., 2015). Pre-pollination mechanisms preclude interspecific pollen flow through changes in habitat use, phenology, floral traits, and breeding systems, and they may evolve in allopatry or under selection against unviable or unfit hybrid progeny (Levin, 2006; Sobel et al., 2010). Post-pollination mechanisms of reproductive isolation generally result from the accumulation of genetic differences during the process of speciation, and they act upon hybrid progeny production, viability, or performance (Orr and Turelli, 2001; Coyne and Orr, 2004). These later barriers have reproductive costs because gametes are wasted and energy is invested in unfit hybrid progeny; thus, pre-pollination RI mechanisms are thought to evolve first and be stronger than post-pollination barriers (Lowry et al., 2008; Baack et al., 2015). However, more studies that jointly evaluate both types of barriers are necessary to determine the prevalence and strength of the different forms of reproductive isolation in lineages of closely related species (Baack et al., 2015).

Habitat isolation is a reproductive barrier caused by local adaptation and limited gene flow among allopatric populations, and it may be one of the first reproductive barriers to evolve in the processes of speciation (Sobel et al., 2010). In the last decade, ecogeographic isolation has been tested using different approaches (e.g., Ramsey et al., 2003; Kay, 2006), but only recently, new methods based on ecological niche modeling have tested whether differences in geographic distributions might also reflect changes in adaptation to particular abiotic environments (Sobel, 2014). When species are not geographically isolated, natural selection should favor the evolution of alternative RI mechanisms.

In some plant groups, flowering phenology plays an important role at preventing interspecific pollen transfer because divergent or sequential flowering times among sympatric congeners allow the temporal separation of pollinator use (e.g., Levin and Anderson, 1970). However, flowering phenology often varies between individuals, populations, and years (Kudo, 2006). Furthermore, within a plant lineage, phenology could be under ecological, developmental, or historical constraints that might restrict variation in the timing of reproductive events (Kochmer and Handel, 1986). In these cases, floral traits related to pollinator attraction may contribute more to reproductive isolation.

Floral isolation may occur through the mechanical coupling between flowers and pollinators or through floral traits that influence pollinator attraction and behavior (Grant, 1994). Specifically, the morphological fit between flowers and pollinators should maximize the precision of contact between floral sexual organs and animal body parts, reducing interspecific pollen transfer (e.g., Pauw, 2006). Floral attractants and rewards also influence pollinator preferences and floral visitation, driving ethological isolation through specific mechanisms of pollen transfer or pollinator constancy (e.g., Schemske and Bradshaw, 1999). The individual contributions of traits involved in floral isolation vary among plant species (Lowry et al., 2008); therefore, it is important to dissect the contribution of different traits to RI.

Changes in plant breeding system have also been associated with the prevention of interspecific pollen transfer in a number of plant groups (e.g., Bromeliaceae, Matallana et al., 2010; *Centaurium*, Brys et al., 2016). Early deposition of self-pollen on stigmas may interfere with deposition of outcross and heterospecific pollen (Brys et al., 2016). A high potential for self-pollination in addition to high levels of natural seed production would indicate that autogamy might also act as a barrier to interspecific pollen transfer in coexisting assemblages of closely related species. Although this topic has received recent attention (Goodwillie and Ness, 2013; Briscoe-Runquist et al., 2014), information on autonomous self-pollination as a mechanism of RI is still sparse relative to our knowledge of other forms of RI.

The family Gesneriaceae provides an interesting system to study the different traits that contribute to reproductive isolation because floral traits vary greatly and many species are habitat specialists that coexist in sympatry (e.g., epiphytic and rupicolous species). Furthermore, many Gesneriaceae species can produce hybrid progeny through horticultural practices and in natural conditions (e.g., Qiu et al., 2011; de Villiers et al., 2013; Smith et al., 2017). Wiehler (1983) proposed that pollinator specialization was one of the main reproductive barriers responsible for maintaining the identity of sympatric congeneric species of Gesneriaceae; however, this idea has not been tested yet.

The aim of this study was to evaluate the importance of different traits involved in the prevention of reproductive interference among three sympatric *Achimenes* species from Mexico. Most members of the genus *Achimenes* occur on wet riparian cliffs in seasonal environments, creating a patchy mosaic of co-existing congeners. While hybrids are commonly generated for the ornamental plant market, hybrids in nature are rare (Ramírez-Roa, 1987; E. Ramírez-Aguirre et al., unpublished data). *Achimenes* is a genus of recent evolutionary origin (Roalson and Roberts, 2016); therefore, it is possible that genetic barriers between species are not fully developed. We evaluated the following reproductive barriers: (1) ecogeographic isolation, (2) phenological isolation, (3) floral isolation and its components (morphology, nectar production and chemical composition, scent production, and pollinator visitation), (4) isolation by self-pollination, and (5) fruit set and seed production as measures of hybrid viability. Barriers 1–5 are considered pre-mating, whereas barrier 5 is post-mating and represents the cost of hybridization at the level of progeny production.

MATERIALS AND METHODS

Study site

The study was conducted in 2013–2016 during the months of July-November at La Tzarazacua Community Park, Parque Nacional Barranca del Cupatitzio, Uruapan, Michoacan, Mexico (19°25′11″–19°26′24″N, 102°07′40″–102°04′20 ″W, 1400–1500 m a.s.l.). Total annual rainfall ranges between 1500–2000 mm, and mean annual temperature is 18–20°C (CONANP, 2006). At the study site, the vegetation includes a combination of pine–oak and cloud forest patches that go from early to mid-late successional stages, intermixed with patches of cattle pasture. *Achimenes* species grow on the outcrops of basaltic rock that characterize the basin and canyons of the Cupatitzio River (CONANP, 2006).

Study species and greenhouse collections

According to the phylogenetic analysis by Roberts and Roalson (2018), *Achimenes* is a genus of Mesoamerican origin that comprises approximately 26 species. *Achimenes antirrhina*, *A. flava*, and *A. patens* belong to a larger clade comprising 10 species, and although these species are not supported as sister to one another, they last shared a common ancestor approximately 4 million years ago (Ma) (Roalson and Roberts, 2016); therefore, they are species of recent divergence. All species are geophytes that inhabit rocky habitats on wet riparian slopes. Geophytes are perennial plants that produce new shoots every spring from underground organs. In the case of *Achimenes*, these shoots reproduce during the growing season and then die, leaving underground rhizomes dormant through the winter. Only two individuals of putative hybrid origin (intermediate corolla shape and color) were found at the study site between *A. flava* and *A. patens*.

Achimenes antirrhina is a short herb, 30–50 cm long with red-orange, tubular flowers that have an inner yellow throat (Fig. 1A). *Achimenes flava* is an herb 20–40 cm long with narrow, bell-shaped, yellow corollas (Fig. 1B). *Achimenes patens* is a small herb 10–30 cm long that has violet, narrow, tubular flowers with an elongated spur (Fig. 1C). All three species are protandrous; anthers dehisce on the first day of anthesis, and stigmas are fully receptive on the fourth day. These species have bilobed or stomatomorphic stigmas (Ramírez-Roa, 1987).

Living specimens of the three study species were grown in greenhouses located at Escuela Nacional de Estudios Superiores, UNAM, Morelia. All specimens were collected as rhizomes and grown in pots with a 1:1:1 mix of sphagnum, rock, and organic matter. To follow natural cycles, watering was started in April and ended in November, when aerial shoots dry out. Rhizomes were kept dry in their pots until the following spring.

Pre-pollination RI: ecogeographic barriers

To assess the role of geographic and habitat isolation in overall RI between the three *Achimenes* species, we analyzed ecogeographic isolation as proposed by Sobel (2014). Using seven bioclimatic layers (four layers that described temperature, and three layers that described precipitation) and a topographic wetness layer with a resolution of 1 km^2, we modeled species distributions in MaxEnt (Phillips and Dúdik, 2008). Using the resulting model layers, we calculated ecogeographic isolation (Eco i) (RI$_2$ equation of Sobel, 2014; see Appendix S1 for full equations and Appendix S2 for full description of species distribution models). In summary, the equation for each species pair was: Eco i = 1 − [shared areas / (shared areas + unshared areas for focal species)].

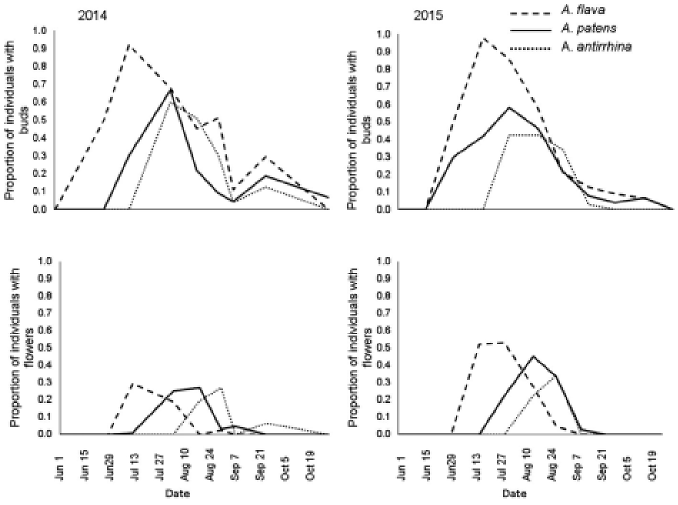

FIGURE 1. Reproductive phenology of three sympatric species of *Achimenes* (Gesneriaceae) monitored at La Tzararacua, Michoacán, Mexico during 2014 and 2015.

Pre-pollination RI: flowering phenology

The flowering phenology of *A. antirrhina*, *A. flava*, and *A. patens* was studied during 2014 and 2015 at La Tzararacua Community Park. In 2014, we marked 60 plants of *A. antirrhina*, 51 of *A. flava*, and 48 of *A. patens*. In 2015, we marked 50 plants of each species. We counted the number of flower buds, open flowers, and mature fruits on each plant every 2 weeks from July to November each year. Since not all plants survived, final sample sizes might differ from initial ones. For this reason, the number of flowering individuals was divided by the number of live individuals on each sampling date.

Differences in flowering phenology between species were tested using circular statistics and the circular package (watson.williams.test function; Agostinelli and Lund, 2017) in R version 3.3.1 (R Core Team, 2018). Circular statistics are appropriate for time data that have an underlying cyclical distribution (Zar, 2014). The Watson–Williams test compares mean angles of two or more samples by first transforming the proportions of flowering individuals to radians; this test assumes a Von Mises distribution. The strength of phenological isolation was evaluated using the 4_{S2} equation from Sobel and Chen (2014; Appendix S1). Total reproductive isolation was calculated using the average of the 2 years when strength values differed by less than 15%. In three cases, yearly values differed by more than 30%; thus, we present the individual values for each year separately.

Pre-pollination RI: floral traits

Floral morphology and color—We evaluated differences in floral morphology between *A. flava*, *A. patens*, and *A. antirrhina* by measuring the following traits on 30 individuals of each species: (1) corolla length, (2) total flower length including corolla and spur, (3) corolla mouth height, (4) corolla mouth width, (5) petal flare, (6) anther height, (7) stigma height, and (8) corolla constriction. Herkogamy was calculated as the difference between anther and stigma height. Since color differences were large, corolla color was assessed qualitatively. To determine the degree of morphological overlap between the study species, first we conducted a principal component analysis (PCA) in R with the stats package (R Core Team, 2018). A multivariate analysis of variance (MANOVA) was used to compare floral phenotypes with traits 1–5 and 8 as dependent variables and species as the main factor. Traits 6 and 7 were not included because *Achimenes* species are protandrous and the length of stamens and style vary with time.

Nectar production and sugar concentration—In the greenhouse, we tagged six flower buds on 13 plants of each *Achimenes* species. We extracted nectar with 1-μL micropipettes through a small hole perforated at the base of the corolla. The length of the nectar column was measured with an analog caliper (DialMax, Willi Hahn Co., Monticello, MN, USA). Sugar concentration was measured with a hand ATC refractometer with temperature calibration and a range of 0–32% °Brix (equivalent to the number of grams of solute per grams of solution). We also analyzed the composition and quantity of nectar sugars using gas chromatography. Nectar was collected in filter paper and eluted in water for posterior gas chromatographic and mass spectrometric analyses as described in Appendix S3. Nectar was measured from 4-d-old flowers, when stigmas were clearly open and turgid.

We used generalized linear models (GLMs) in the stats package of R (R Core Team, 2018) to test for differences between species (predictor variable) in mean nectar volume per flower (response variable; gamma distribution and inverse link function). We also tested for differences in sugar concentration between species, with different error distributions depending on the nature of data (total concentration: gamma with inverse link; sucrose and glucose: Gaussian with identity link; fructose: inverse Gaussian with $1/mu^2$ link; inositol: Poisson with log link; °Brix concentration: quasibinomial with log link). Analyses were performed with the stats package in R (R Core Team, 2018).

Floral scents—Floral volatiles were quantified in the laboratory in four individuals per species, which had been collected in the field and kept in a live collection in the greenhouses of ENES-Morelia, Universidad Nacional Autónoma de México. Volatiles were extracted from flowers that were enclosed in glass jars by vacuuming air for 7 h (from 08:00 to 15:00 hours) and eluted in hexane. After elution, volatiles were analyzed with gas chromatography. Detailed methods are described in Appendix S3.

Pre-pollination RI: Pollinator visitation

Floral visitors at focal plants or plant patches of each species were directly observed and recorded with video cameras (SONY, Japan, and Panasonic, Japan) for 1-h periods throughout the day (08:00–16:00 hours) in sunny to cloudy-misty weather, during the flowering seasons of 2013–2015. Total observation times and sample sizes are provided with pollinator visitation results. We recorded time of visitation, identity and behavior of the visitor, and number of flowers probed. Visitors that contacted the reproductive organs of the flowers were considered legitimate pollinators. We calculated pollinator visitation rates by pollinator functional group (i.e., hummingbird, large bee, small bee, butterfly) as the mean number of visits per flower per hour. Since visitation rates were low and collection of all floral visitors was not possible, we identified most pollinators to the lowest possible taxonomic category from video recordings and photographs. We searched the literature for information on length of mouthparts (i.e., proboscis or beak) of the different pollinator groups to assess their association with corolla length. Reproductive isolation was calculated using RI_{4A} equation of Sobel and Chen (2014; Appendix S1).

Pre-pollination RI: autonomous self-pollination

We conducted a greenhouse experiment in 2015 to evaluate the role of self-pollination as a potential RI mechanism. We marked three flower buds per plant on 24 individuals of each species and assigned them to one of the following treatments: (1) autonomous pollination (unmanipulated bagged flowers), (2) hand cross-pollination (emasculated flowers; pollen from two donors of the same species, i.e., intraspecific crosses), and (3) hand self-pollination (pollen from flowers of the same plant). We previously assessed time of stigma receptivity by recording peroxidase activity each day of anthesis (Kearns and Inouye, 1993), which corresponded to full extension of stigma lobes in all species. Mixtures of pollen from four fresh stamens were placed directly onto stigmas, ensuring stigma surfaces were saturated with pollen. To compare the fruit set of autonomous self-pollination with the fruit set achieved under natural conditions, in the field we quantified the fruit set from unmanipulated flowers (one per plant in 30 plants per species). For all treatments, we followed fruit development until maturity and collected dry mature capsules prior to dehiscence.

We used generalized linear mixed models (GLMMs) in the lme4 package in R program (Bates et al., 2015; R Core Team, 2018) to determine the effect of treatment on fruit set (binomial, logit link function). Individual was included as a random factor. Contrasts between treatments were performed with glht function and single-step method in package multcomp (Hothorn et al., 2008).

Post-pollination RI: hybrid viability

In the greenhouse, we conducted intraspecific and interspecific crosses in 2016 and 2017 to evaluate post-pollination barriers to hybrid fruit and seed production and potential costs of hybridization. We tagged 33 individual plants of *A. antirrhina*, 29 of *A. flava*, and 52 of *A. patens*; however, final sample sizes varied from loss of flower buds or individuals during the study. Three flower buds per individual were assigned to a different hand-pollination treatment, where each plant served as a pollen donor and a pollen recipient for each of two congeners. Pollen was saturated onto stigmas using anthers of the paternal species. Approximately 2 months after pollination, we counted and collected dry mature capsules. Capsules were immediately placed in petri dishes until they released the seeds. We took photographs of all seeds produced by each fruit with a Stemi 350 stereoscope and an Axiocam 105-color (Carl Zeiss, Germany) and counted viable and nonviable seeds. Preliminary work indicated that aborted seeds have contorted shapes and smaller sizes; therefore, we assessed seed shape and size to estimate the total number of viable seeds produced per species (hereafter referred to as seed production). For analyses, we used fruit set (fruits/flowers) and seed production as proxies of hybrid viability.

After inspecting residuals under a linear model, we used GLMMs in the lme4 package (Bates et al., 2015; R Core Team, 2018) to test for the effect of treatment (intraspecific and both interspecific crosses) on fruit set (binomial distribution, logit link function) and seed production (Poisson distribution, log link function). Individual plant was included as a random factor. Ad hoc comparisons of the intraspecific pollination treatment vs. each interspecific cross were computed with a two-tailed test, using multcomp with single-step method and multiple comparisons package in R (Hothorn et al., 2008; R Core Team, 2018). Data for 2016 and 2017 were pooled because sample sizes in 2016 were small (less than 13) and fruit set values were similar in both years. For calculations of reproductive isolation at the level of fruit set and seed production, we used the RI_{4A} equation from Sobel and Chen (2014), which considers the probability of gene flow between species pairs (Appendix S1). RI values range from 1 (complete isolation) to −1 (complete disassortative mating; RI = 0 indicates no isolation; Sobel and Chen, 2014).

Total reproductive isolation

To understand the contributions of each reproductive barrier (ecogeographic isolation, phenological isolation, floral isolation, and hybrid fruit set and seed production) to total reproductive isolation, we used the RI$_{4E}$ equation and calculations proposed by Sobel and Chen (2014). Detailed methods are presented in Appendix S1. We show the individual strengths of each barrier and the absolute contributions of each barrier to total isolation excluding ecogeographic isolation (to obtain RI estimates under sympatric conditions).

RESULTS

Pre-pollination RI: ecogeographic barriers

The three study species were found in both sympatric and allopatric populations. Ecogeographic isolation values between species pairs were over 0.6 in all cases (Table 1). The more ecogeographically isolated species pair was *A. patens* and *A. antirrhina* (0.74) and the least isolated *A. antirrhina* and *A. flava* (0.64).

Pre-pollination RI: flowering phenology

The flowering times of the three study species overlapped during August and September, but initial flowering dates and peaks differed (Fig. 1). There were no significant differences in mean flowering times between species in 2014 (Watson–Williams test $F_{2, 18} = 0.03$, $P = 0.970$), nor in 2015 ($F_{2, 21} = 0.57$, $P = 0.574$). Mean flowering times corresponded to the first 2 weeks of August in 2014 and mid August in 2015. RI due to flowering phenology varied between species pairs and was higher in 2014 than in 2015 (RI range, 2014: 0.43–0.94, 2015: 0.09–0.70; Table 1).

Pre-pollination RI: floral traits

Floral morphology—Floral phenotypes differed considerably between species and showed no overlap in morphological space (MANOVA, $F_{12, 160} = 170.5$, $P = 2.2 \times 10^{-16}$; Fig. 2; Appendix S4). The traits that contributed most to variation were corolla length, total flower length, and corolla mouth length for PC axis 1 (56.4% of total variance) and corolla mouth width and petal flare to PC

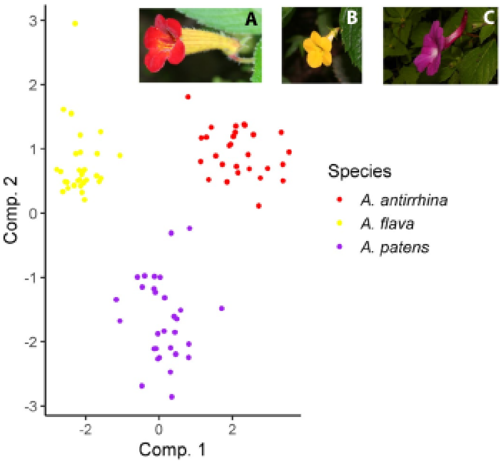

FIGURE 2. Principal component analysis of floral traits for three sympatric species of *Achimenes* from La Tzararacua, Michoacán, Mexico. *Achimenes antirrhina* (A), *A. flava* (B), *A. patens* (C).

TABLE 1. Strength of reproduction isolation for individual barriers and total isolation values between three *Achimenes* species from Central Mexico. Absolute contributions of each barrier to total isolation include all barriers (AC) and barriers acting in sympatry (AC-Sym). Relative contributions to total isolation (not shown) equaled ACs, except for the *A. patens* × *A. flava* cross.

Maternal × Paternal	*A. antirrhina* × *A. flava*			*A. antirrhina* × *A. patens*		
Barrier	Strength	AC	AC-Sym	Strength	AC	AC-Sym
Ecogeographic	0.638	0.64	—	0.743	0.74	
Phenological[a]	0.830	0.30	0.83	0.460, 0.149	0.12, 0.04	0.46, 0.15
Floral	1	0.06	0.17	1	0.14, 0.22	0.54, 0.85
Fruit set	0.660	0	0	0.560	0	0
Total		**1**	**1**		**1**	**1**
		A. flava* × *A. antirrhina			***A. flava* × *A. patens***	
Ecogeographic	0.638	0.64	—	0.716	0.72	
Phenological[a]	0.923, 0.493	0.33, 0.18	0.92, 0.49	0.432	0.12	0.43
Floral	1	0.03, 0.18	0.08, 0.51	1	0.16	0.57
Fruit set	0.700	0	0	−0.006	0	0
Seed production	0	0	0	0.35	0	0
Total		**1**	**1**		**1**	**1**
		A. patens* × *A. antirrhina			***A. patens* × *A. flava***	
Ecogeographic	0.743	0.74 /0.74	—	0.715	0.72	—
Phenological[a]	0.429, 0.093	0.11, 0.02	0.43, 0.09	0.494	0.14	0.49
Floral	1	0.15, 0.24	0.57, 0.91	0.800	0.11	0.40
Fruit set	0.210	0	0	0.089	0.01	0.02
Seed production	0.500	0	0	0.292	0.01	0.04
Total		**1**	**1**		**0.99**	**0.95**

[a] Mean values for 2014 and 2015 phenological reproductive isolation were used for yearly strength values that differed by less than 15%. Individual values for each year are given and separated by a comma when differences exceeded 30.

axis 2 (28.2% of total variance). Corolla lengths coincided with the mouthparts of the main pollinators of each *Achimenes* species (Table 2). Corolla color also differed between species (Fig. 2).

Nectar production and sugar composition—Nectar volume differed among the three *Achimenes* species (χ^2 = 46.3, df = 2, $P = 8.9 \times 10^{-11}$; Table 3); the highest volume was for ornithophilous *A. antirrhina* (6.6 ± 47.62 µL), and the lowest was for *A. flava* (0.8 ± 5.59 µL). Sucrose was the most abundant nectar sugar in all three species, ranging between 107 and 135 µg/µL (Table 3) and did not differ in concentration among *Achimenes* species (χ^2 = 1.4, df = 2, P = 0.503). Hexoses (fructose and glucose) and inositol were three orders of magnitude lower than sucrose; fructose and glucose concentration differed among species (χ^2 = 7.1, df = 2, P = 0.028; χ^2 = 6.0, df = 2, P = 0.049, respectively), while inositol concentration did not (χ^2 = 0.17, df = 2, P = 0.917; Table 3). Total sugar concentration ranged between 108 and 159 µg/µL and did not differ among species (χ^2 = 1.3, df = 2, P = 0.517; Table 3), neither did sugar concentration expressed as °Brix (χ^2 = 2.6, df = 2, P = 0.276).

Floral scents—Organic volatiles in flowers included the terpenoids pinene, limonene, cineole, the benzenoid naphthalene, and the fatty-acid derived alkene tetradecane, but the presence and concentration of these compounds varied among species (Table 4). The floral scent profile of *A. antirrhina* showed two of the five volatiles, while the profile of *A. flava* and *A. patens* showed four of the five volatile compounds. The last two species differed in one volatile compound; limonene was exclusive to *A. patens*, and cineole was exclusive to *A. flava* (Table 4).

Pre-pollination RI: pollinator visitation

The main pollinators of the study species were hummingbirds for *A. antirrhina*, bees for *A. flava*, and butterflies for *A. patens* (Table 5). *Amazilia beryllina* was the only hummingbird species that visited *A. antirrhina*, and it was not observed at any other plant species at the study site. This hummingbird generally probed various flowers within a patch, but territorial behavior was not observed. Bee visitors to flowers of *A. flava* included medium-sized bees (tribes Centridini and Eucerini), small halictid bees and one crabronid wasp species; a butterfly from the genus *Pieris* visited flowers once in 2013. All lepidopterans observed at *A. patens* were in the families Hesperiidae and Pieridae. Thrips and the Crabronidae wasp were also observed visiting *A. patens*. Thrips acted as nectar robbers because they did not contact the reproductive organs of the flowers, while Crabronid wasps may be occasional pollinators. Reproductive isolation through pollinator visitation was complete for most species pairs except for *A. patens*–*A. flava* (Table 1).

Pre-pollination RI: autonomous self-pollination

All three species are self-compatible since they produce fruit and seed after hand-self pollination (Fig. 3). However, the values of autonomous fruit set ranged between 0 and 4%, while hand-cross pollination consistently resulted in fruit set higher than 50% (Fig. 3); thus, autonomous self-pollination does not contribute to RI. Fruit set from hand cross- and self-pollination were higher than fruit set from natural pollination (*A. antirrhina*: χ^2 = 5.9, df = 2, P = 0.049; *A. flava*: χ^2 = 60.0, df = 3, P < 0.0001; *A. patens*: χ^2 = 57.1, df = 3, P < 0.0001).

TABLE 2. Mean (±SEM) corolla length and corolla mouth width of three sympatric *Achimenes* species from Central Mexico, and bill or proboscis length (range) of their primary pollinators recorded from the literature.

Species	*N*	Corolla length (mm)	Corolla width (mm)	Main pollinator	Bill/ proboscis length (mm)	Source
A. antirrhina	30	27.3 ± 0.70	6.13 ± 0.14	*Amazilia beryllina*	18-21	Howell, 2003
A. flava	30	10.1 ± 0.15	5.72 ± 0.17	*Centris* aff. *atripes*	11-14^{a}	Roubik et al., 1995
A. patens	30	14.5 ± 0.25	3.20 ± 0.13	*Urbanus* sp.	16-17	Bauder et al., 2015

^{a}Proboscis lengths of other *Centris* species collected in Mexico.

TABLE 3. Mean (±SEM) floral nectar volume and sugar concentration for three species of *Achimenes* from Central Mexico. Nectar samples were obtained from greenhouse plants previously collected at La Tzararacua, Michoacán.

Species	*N*	Nectar vol. (µL)	Fructose (µg/µL)	Glucose (µg/µL)	Sucrose (µg/µL)	Inositol (µg/µL)	S/H (µg/µL)	Total (µg/µL)	°Brix (%)
A. antirrhina	11	6.6 ± 1.04^{a}	0.5 ± 0.64^{a}	0.02 ± 0.014^{b}	136 ± 28.7^{a}	0.2 ± 0.11^{a}	805 ± 328.5	136 ± 31.3^{a}	33 ± 3.8^{a}
A. flava	10	0.8 ± 0.11^{b}	0.2 ± 0.32^{b}	0.07 ± 0.015^{a}	107 ± 32.7^{a}	0.2 ± 0.15^{a}	621 ± 216	108 ± 27.0^{a}	40 ± 3.2^{a}
A. patens	13	3.2 ± 0.4^{c}	0.2 ± 0.30^{b}	0.03 ± 0.014^{b}	158 ± 28.7^{a}	0.2 ± 0.13^{a}	835 ± 189.7	159 ± 29.46^{a}	33 ± 3.7^{a}

Notes: Nectar volume (vol), raw values for fructose and sugar concentration are given for easier comparison. S/H, proportion of sucrose (S) to common hexoses (H = fructose + glucose); °Brix, sugar concentration (expresses mass/mass relation). Different letters within a column indicate a difference between species.

TABLE 4. Mean relative percentage (±SEM) of floral scent compounds of three *Achimenes* species collected at La Tzararacua site and grown in a greenhouse.

Compound	KRI	*A. antirrhina*	*A. flava*	*A. patens*
α-Pinene	939	58.0 ± 1.78	52.4 ± 17.30	38.5 ± 20.91
Limonene	1029	—	—	13.6 ± 7.88
1,8-Cineole	1031	—	5.0 ± 5.03	—
cis-Decahydronaphthalene	1099	41.9 ± 1.78	20.2 ± 9.31	28.8 ± 18.99
Tetradecane	1400	—	22.3 ± 13.71	17.0 ± 9.82

Note: KRI, Kovats retention index.

TABLE 5. Mean pollinator visitation rates (±SEM) of three *Achimenes* species at La Tzararacua, Michoacán, Mexico during 2013–2015, calculated as mean number of visits $flower^{-1}\ h^{-1}$. *N* is the total number of observation hours.

		Mean number of visits $flower^{-1}\ h^{-1}$		
Species	**Pollinator taxon**	**2013 (*N* = 12)**	**2014 (*N* = 10)**	**2015 (*N* = 5, 6, 8)[a]**
A. antirrhina	**Trochilidae** *Amazillia beryllina*	0.10 ± 0.011	0.19 ± 0.012	0.6 ± 0.4
A. flava	**Hymenoptera** *Centris* aff. *atripes* (Apidae) Eucerini sp. Halictidae (1 sp.) Crabronidae (1 sp) *Pieris* sp.	0.14 ± 0.032	0.23 ± 0.091	0.16 ± 0.17
A. patens	**Lepidoptera** *Urbanus dorantes*, *U. proteus* (Hesperiidae) Pieridae (aff. *Pieris* sp.)	0.34 ± 0.140	0.12 ± 0.07	0.5 ± 0.26
	Hymenoptera Crabronidae (1 sp.)			0.25 ± 0.25

[a] Sample sizes, respectively, for *A. antirrhina*, *A. flava*, *A. patens*.

Post-pollination RI: hybrid viability

Interspecific crosses produced fruits in eight of nine parental combinations; however, fruit set was asymmetric between crosses according to the identity of the donor and recipient species (Figs. 4,5). Fruit set ranged between 53 and 75% for intraspecific crosses and between 11 and 76% for interspecific crosses (Fig. 4). When *A. antirrhina* was the pollen recipient, hybrid fruit set was lower than intraspecific fruit set in both interspecific crosses ($\chi^2 = 10.5$, df = 2, $P = 0.005$). When *A. flava* was the pollen recipient, hybrid fruit set was significantly lower than intraspecific fruit set only when crossed with *A. antirrhina* ($\chi^2 = 21.8$, df = 2, $P < 0.0001$). When *A. patens* was the pollen recipient, hybrid fruit set values were lower, but they did not significantly differ from intraspecific fruit set ($\chi^2 = 5.2$, df = 2, $P = 0.075$). Reproductive isolation estimates for interspecific crosses ranged from –0.006 for the cross *A. flava* × *A. patens* to 0.70 for the *A.* flava × *A. antirrhina* cross (Table 1).

Seed production varied with the identity of the pollen recipient and donor species. Seed production ranged between 304 and 946 for intraspecific crosses and between 146 and 311 for hybrid crosses, and the cross *A. flava* × *A. antirrhina* did not yield any viable seed (Fig. 5). For both *A. flava* and *A patens* as pollen recipients, hybrid seed production values were significantly lower than intraspecific seed production ($\chi^2 = 367.8$, df = 1, $P < 0.0001$; $\chi^2 = 3075.3$, df = 2, $P < 0.0001$, respectively). RI values ranged from 0.29 (*A. patens* × *A. flava*) to 0.5 (*A. patens* × *A.antirrhina*). Crosses with *A. antirrhina* as a maternal plant were not assessed due to high fruit mortality.

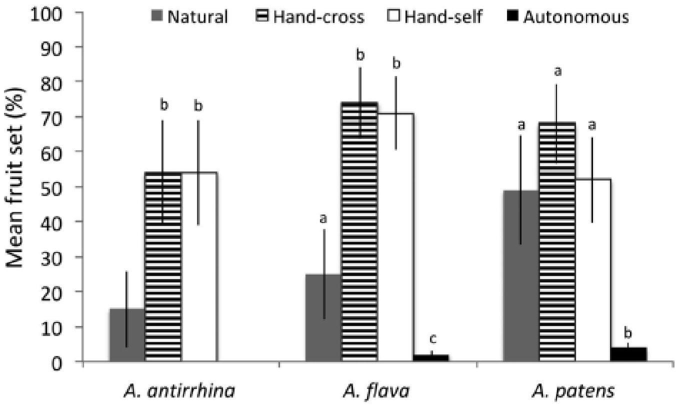

FIGURE 3. Mean fruit set (±SEM) from four pollination treatments used to determine the capacity for autonomous self-pollination in three sympatric species of *Achimenes* from Central Mexico. Hand pollinations were done in the greenhouse and natural pollination was quantified at La Tzararacua, Michoacán, during 2015. Letters indicate significant differences between treatments within a species.

DISCUSSION

Pre- and post-mating mechanisms of reproductive isolation

This study evaluated different plant traits that might be involved in reproductive isolation among three congeneric sympatric Gesneriaceae species. *Achimenes flava*, *A. antirrhina*, and *A. patens* are habitat specialists that occur on humid rocky slopes of river canyons in seasonal environments in the mountains of central Mexico; thus, we predicted they would often coexist in such microhabitats. However, the results showed that ecogeographic isolation is an important pre-pollination barrier when estimated from relatively large geographic scales (1-km^2 resolution layers), indicating that sympatric assemblages are not as common as expected and that sympatric sites should be hotspots of selection for other pre-pollination mechanisms of RI.

Flowering phenology largely overlapped between species at a site of sympatry; however,

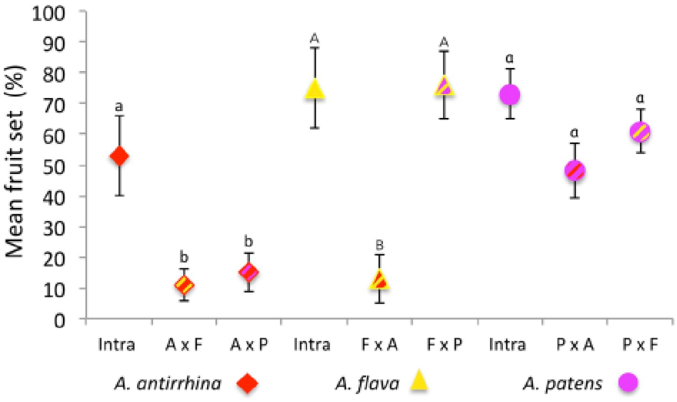

FIGURE 4. Mean fruit set (±SEM) obtained from intra- and interspecific crosses between three *Achimenes* species from Central Mexico. Letters indicate species and shapes indicate maternal species for each cross: A /diamond = *A. anthirrhina*, F /triangle = *A. flava*, P /circle = *A. patens*. Letters over symbols indicate significant differences between treatments within each maternal species.

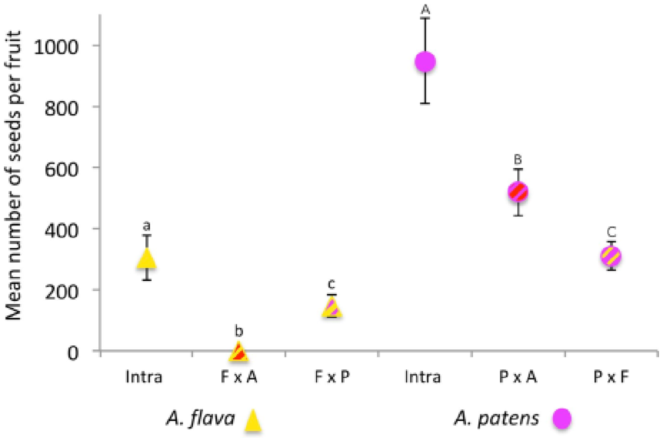

FIGURE 5. Mean seed production per fruit (±SEM) obtained from intra- and interspecific crosses between three *Achimenes* species from Central Mexico. Letters indicate species and shapes indicate maternal species for each cross: A /diamond = *A. anthirrhina*, F /triangle = *A. flava*, P /circle = *A. patens*. Letters over symbols indicate significant differences between treatments within each maternal species.

flowering peaks differed significantly, particularly in 2014. In most cases, flowering phenology was an important contributor to RI, but it varied between years and species pairs. The overlapping flowering seasons of the study species may be associated with their pseudo-annual life history, with aboveground stems that die and regrow every year from underground rhizomes at the beginning of the rainy season. This life cycle restricts the time available for growth and reproduction, as has been described for other geophytic plant species (Dafni et al., 1981). Hence, if closely related sympatric species are constrained to flower during the same period of the year, selection may favor floral traits that promote specialization in pollination systems (Rathcke and Lacey, 1985).

Floral isolation is, according to our results, the most important reproductive barrier among the study species. Only twice during the study, the same pollinator was observed visiting flowers of two *Achimenes* species, which suggests that particular floral traits attract certain kinds of floral visitors and deter others (see discussion below). Floral isolation through morphology (mechanical isolation) is one of the most important RI mechanism among sympatric species in various plant genera (e.g., *Asclepias*, Kephart and Theiss, 2003; *Costus*, Kay, 2006; *Spiranthes sinensis* complex, Tao et al., 2018), but ethological isolation through signals and rewards that influence pollinator behavior are also important (Schemske and Bradshaw, 1999; Klahre et al., 2011; Byers et al., 2014).

Autogamy may act as a reproductive barrier in some species (Levin, 1971; Brys et al., 2016), but it is unlikely to be relevant in *Achimenes*, given the low fruit set resulting from autonomous self-pollination. Furthermore, post-zygotic barriers were weak in most species pairs, indicating that reproductive isolation is incomplete at the level of hybrid viability, a finding that has been reported for other Gesneriaceae species (Johnson et al., 2015; Zhang et al., 2017). Fruit and seed production from interspecific crosses varied according to the identity of the pollen recipient species, and they were often asymmetrical (Fig. 3). These results may reflect the inability of pollen tubes from smaller flowers (i.e., *A. flava*, *A. patens*) to develop past their autotrophic phase down to the ovaries of the long-styled *A. antirrhina*, but this idea needs to be tested. Other mechanisms that explain asymmetries in hybrid viability in other species are genetic and intracellular incompatibilities, pollen–pistil interactions, triploid endosperm interactions and/or maternal effects (Turelli and Moyle, 2007). Our results of hybrid viability, estimated from fruit and seed production are congruent with tests of hybridization, where several Gesneriaceae species have a high potential to produce hybrid progeny; nonetheless, pollen viability may be reduced in some *Achimenes* hybrids, indicating barriers may be acting at the level of hybrid fertility (Cooke and Lee, 1966; Wiehler, 1983).

Extrinsic mechanisms that act on individual hybrids, such as low competitiveness in the parental environment might also account for the low occurrence of hybrid phenotypes in the wild (Widmer et al., 2009). We registered two individuals with floral phenotypes that were intermediate between *A. flava* and *A. patens* in 2013, but we did not find them the following years. Interestingly, pollen viability in hybrids from *A. flava* and *A. patens* is less than 30% (Wiehler, 1983), suggesting that even if hybrid fruits are produced, hybrid individuals are possibly poor pollen donors. Overall, findings from this and other studies suggest that there is a cost involved in the production of hybrid progeny.

Floral features that contribute to reproductive isolation—Different floral morphologies in closely related species may promote intraspecific pollination by placing pollen differentially on the bodies of particular pollinators or by attracting specific functional groups of pollinators (e.g., Pauw, 2006; Martén-Rodríguez et al., 2009). Two of the three study species, *A. antirrhina* and *A. patens*, have floral morphologies that promote effective pollen transfer by a single functional group of pollinators and restrict access to unwanted visitors (Fig. 2). Narrow, long corollas and spurs in *A. patens* only allow access to nectar to insects with relatively long proboscides, such as the observed hesperid butterflies (see Table 2). A similar association has been described for various plant groups pollinated by lepidopterans or long-tongued flies (e.g., Whittall and Hodges, 2007; Pauw et al., 2009). Likewise, in the case of *Achimenes antirrhina*, corollas are tubular and only a few millimeters longer than the mouthparts of their hummingbird pollinators (*Amazilia beryllina* bill length: 19–21 mm; Howell, 2003). This type of mechanical isolation has been described for various plant taxa, such as the genera *Aquilegia*, *Penstemon*, and *Costus* (e.g., Fulton and Hodges, 1999; Castellanos et al., 2004; Kay, 2006), although the flowers of hummingbird-pollinated plants are often wide enough to be visited by bees and other small insects. In the case of *A. antirrhina*, the absence of bees might be associated with reduced attraction due to flower color; however, an assessment of flower color spectra and color vision of local bees would be necessary to test this idea.

The manipulation of pollinator behavior based on pollinator senses, feeding preferences and energetic demands may contribute to preventing heterospecific visitation (i.e., ethological isolation, Grant, 1994). For example, differences in pollinator attraction mediated by the quantity or quality of floral rewards may play an important role at preventing interspecific pollen transfer (Mitchell, 1993; Schemske and Bradshaw, 1999). In this study, the nectar volume produced per flower differed between the three *Achimenes* species consistent with previous studies (Baker and Baker, 1983; Cruden et al., 1983), higher volumes were produced by hummingbird-pollinated *A. antirrhina* and the lowest volumes by bee-pollinated *A. flava*. Nectar sugar composition has also been shown to differ according to the preferences of particular pollinators (Baker and Baker, 1983). However, in the present study, nectar composition did not contribute to floral isolation because sucrose was the dominant sugar in the nectar of all three *Achimenes* species and the content of other sugars varied little among species. These results suggest that nectar sugar composition is phylogenetically conserved in *Achimenes*, similar to the case of hummingbird- and bee-pollinated species of the Brazilian clade Sinningiae (Gesneriaceae; Perret et al., 2001).

In the case of floral scents, three volatiles were shared between the insect-pollinated species, and two of these were present in hummingbird-pollinated *A. antirrhina*. The remaining volatiles included one compound that was exclusive to bee-pollinated *A. flava* (1,8-cineole) and one exclusive to butterfly-pollinated *A. patens* (limonene). The finding of only two floral volatiles in *A. antirrhina* agrees with findings for other ornithophilous species and suggests that nearly scentless flowers evolve in lineages pollinated by hummingbirds (Knudsen et al., 2004), although sense of smell has been little studied in these vertebrates (e.g., Goldsmith and Goldsmith, 1982). In contrast, visual cues and floral scents are common in bee-pollinated species (Dobson, 2006). Interestingly, of the four scents in *A. flava*, 1,8-cineole and α-pinene are commonly found in flowers pollinated by male euglossine bees, and tetradecane is common in flowers pollinated by nectar-seeking bees (Gerlach and Schill, 1991; Dobson, 2006; Martel et al. 2019). In contrast, terpenoids such as 1,8-cineole and limonene are apparently not well perceived by butterflies (Andersson and Dobson, 2003); thus, the function of limonene in flowers of *A. patens* needs to be further explored. Finally, the unexpected presence of naphthalene in all species may be an herbivore deterrent, as suggested for *Magnolia* (Azuma et al., 1996). Future studies should address the association between volatiles and pollination systems to determine their potential role in reproductive isolation in the Gesneriaceae.

CONCLUSIONS

Our results highlight the importance of pollination system specialization at preventing interspecific pollination and avoiding the costs of hybrid progeny production, allowing the co-occurrence of closely related species with reduced reproductive interference. We determined that mechanical (floral morphology) and ethological traits (nectar volume and floral volatiles) contribute to promote intraspecific visitation by particular pollinators. In contrast, phenology does not allow full temporal separation of reproductive seasons and post-zygotic barriers are weak, generating conditions that might favor pollinator-mediated selection on floral traits to reduce reproductive interference. These results suggest a potential role of reinforcement (i.e., selection that acts on particular traits to reduce the costs associated with the production of hybrids of low viability or performance; Hopkins, 2013) in the floral diversification of the genus *Achimenes*. Future research investigating the role of phylogenetic constraints on phenological and nectar traits and the role of reinforcement in the diversification of floral traits in tropical geophytes is warranted.

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AUTHOR CONTRIBUTIONS

E.R.A. and S.M.R. designed the study, collected and analyzed data, and wrote the manuscript. Y.M.D., F.J.E.G. and G.Q.A. analyzed nectar and floral scent data; G.Q.A. and Y.M.D. conducted interspecific pollinations; and M.Q. and K.O. contributed to fieldwork and manuscript writing.

DATA ACCESSIBILITY

Calculations of reproductive isolation were based on supporting information from Sobel and Chen (2014) at https://onlinelibrary.wiley.com/doi/full/10.1111/evo.12362. Species presence data and geographic information were accessed from www.gbif.org; http://www.worldclim.org/version1; http://www.conabio.gob.mx/informacion/gis/; and https://web.archive.org/web/20170519221949/http://worldgrids.org/doku.php/wiki:layers#dem-derived_parameters.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Reproductive isolation equations from Sobel and Chen (2014) used in the analysis of reproductive barriers among three *Achimenes* species from Central Mexico.

APPENDIX S2. Methods used to estimate ecogeographic isolation between three *Achimenes* species from Central Mexico.

APPENDIX S3. Methods for nectar and scent analyses used for three *Achimenes* species from Central Mexico.

APPENDIX S4. Floral trait measurements of three *Achimenes* species from La Tzararacua, Michoacán, México.

LITERATURE CITED

Agostinelli, C., and U. Lund. 2017. R package circular: circular statistics (version 0.4-93). Available at https://r-forge.r-project.org/projects/circular/.

Andersson, S., and H. E. M. Dobson. 2003. Antennal responses to floral scents in the butterfly *Heliconius melpomene*. *Journal of Chemical Ecology* 29: 2319–2330.

Azuma, H., M. Toyota, Y. Asakawa, and S. Kawano. 1996. Naphthalene- a constituent of *Magnolia* flowers. *Phytochemistry* 42: 999–1004.

Baack, E., M. C. Melo, L. H. Rieseberg, and D. Ortíz-Barrientos. 2015. The origins of reproductive isolation in plants. *New Phytologist* 207: 968–984.

Baker, H. G., and I. Baker. 1983. Floral nectar constituents in relation to pollinator type. *In* C. E. Jones and R. J. Little [eds]. Handbook of pollination biology, 117–141. Scientific and Academic Editions, Van Nostrand Reinhold, NY, NY, USA.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Bauder, J. A. S., A. D. Warren, and H. W. Krenn. 2015. The ecological role of extremely long-proboscid Neotropical butterflies (Lepidoptera:Hesperiidae) in plant-pollinator networks. *Arthropod-Plant Interactions* 9: 415–424.

Briscoe-Runquist, R. D., E. Chu, J. L. Iverson, J. C. Kopp, and D. A. Moeller. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* 68: 2885–2900.

Brys, R., J. van Cauwenberghe, and H. Jacquemyn. 2016. The importance of autonomous selfing in preventing hybridization in three closely related plant species. *Journal of Ecology* 104: 601–610.

Byers, K. J. R. P., H. D. Bradshaw, and J. A. Riffell. 2014. Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *Journal of Experimental Biology* 217: 614–623.

Castellanos, M. C., P. Wilson, and J. D. Thomson. 2004. 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *Journal of Evolutionary Biology* 17: 876–885.

CONANP [Comision Nacional de Áreas Naturales Protegidas]. 2006. Programa de Conservación y Manejo del Parque Nacional Barranca del Cupatitzio. CONANP, Ciudad de México, México. Available at https://simec.conanp.gob.mx/pdf_libro_pm/3_libro_pm.pdf.

Cooke, J. F., and R. E. Lee. 1966. Hybridization within and between *Achimenes* P. Br. and *Smithiantha* Kuntze (Gesneriaceae). *Baileya* 14: 92–101.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer, Sunderland, MA, USA.

Cruden, R. W., S. M. Herman, and S. Paterson. 1983. Patterns of nectar production and plant-pollinator coevolution. *In* B. Bentley and T. S. Ellias [eds.], The biology of nectaries, 80–125. Columbia University Press, NY, NY, USA.

Dafni, A., D. Cohen, and I. Noi-Mier. 1981. Life-cycle variation in geophytes. *Annals of the Missouri Botanical Garden* 68: 652–660.

Dobson, H. E. M. 2006. Relationship between floral fragrance composition and type of pollinator. *In* N. Dudareva and E. Picherskyeds [eds.], Biology of floral scent, 147–198. CRC Press, Boca Raton, FL, USA.

de, Villiers, M.J., M. D. Pirie, M. Hughes, M. Möller, T. J. Edwards, and D. U. Bellstedt. 2013. An approach to identify putative hybrids in the "coalescent stochasticity zone", as exemplified in the African plant genus *Streptocarpus* (Gesneriaceae). *New Phytologist* 198: 284–300.

Fulton, M., and S. A. Hodges. 1999. Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the Royal Society, B, Biological Sciences* 266: 2247–2252.

Gerlach, G., and R. Schill. 1991. Composition of orchid scents attracting euglossine bees. *Botanica Acta* 104: 379–384.

Goldsmith, K. M., and H. Goldsmith. 1982. Sense of smell in the black-chinned hummingbird. *Condor* 84: 237–238.

Goodwillie, C., and J. N. Ness. 2013. Interactions of hybridization and mating systems: a case study in *Leptosiphon* (Polemoniaceae). *American Journal of Botany* 100: 1002–1013.

Grant, V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences, USA* 91: 3–10.

Hopkins, R. 2013. Reinforcement in plants. *New Phytologist* 197: 1095–1103.

Hopkins, R., and M. D. Rausher. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. *Science* 335: 1090–1092.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. *Biometrical Journal* 50: 346–363.

Howell, S. N. G. 2003. Hummingbirds of North America: the photographic guide: 70. Princeton University Press, Princeton, NJ, USA.

Johnson, M. A., D. K. Price, J. P. Price, and E. A. Stacy. 2015. Post-zygotic barriers isolate sympatric species of *Cyrtandra* (Gesneriaceae) in Hawaiian montane forest understories. *American Journal of Botany* 102: 1870–1882.

Kay, K. M. 2006. Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution* 60: 538–552.

Kearns, C. A., and D. W. Inouye. 1993. Techniques for pollination biologists, 1st ed. University Press of Colorado, Boulder, CO, USA.

Kephart, S., and K. Theiss. 2003. Pollinator mediated isolation in sympatric milkweeds (*Asclepias*): Do floral morphology and insect behavior influence species boundaries? *New Phytologist* 161: 265–267.

Klahre, U., A. Gurba, K. Hermann, M. Saxenhofer, E. Bossolini, P. M. Guerin, and C. Kuhlemeier. 2011. Pollinator choice in *Petunia* depends on two major genetic loci for floral scent production. *Current Biology* 21: 730–739.

Knudsen, J. T., L. Tollsten, I. Groth, G. Bergström, and R. A. Raguso. 2004. Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. *Botanical Journal of the Linnean Society* 146: 191–199.

Kochmer, J. P., and S. N. Handel. 1986. Constraints and competition in the evolution of flowering phenology. *Ecological Monographs* 56: 303–325.

Kudo, G. 2006. Flowering phenologies of animal-pollinated plants: reproductive strategies and agents of selection. *In* L. D. Harder and S. C. H. Barrett [eds.], Ecology and evolution of flowers, 139–156. Oxford University Press, Oxford, UK.

Kyogoku, D. 2015. Reproductive interference: ecological and evolutionary consequences of interspecific promiscuity. *Population Ecology* 57: 253–260.

Levin, D. A. 1971. The origin of reproductive isolation mechanisms in flowering plants. *Taxon* 20: 91–113.

Levin, D. A. 2006. Flowering phenology in relation to adaptive radiation. *Systematic Botany* 31: 239–246.

Levin, D. A., and W. W. Anderson. 1970. Competition for pollinators between simultaneously flowering species. *American Naturalist* 104: 455–467.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu, and J. H. Willis. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society, B, Biological Sciences* 363: 3009–3021.

Martel, C., G. Gerlach, M. Ayasse, and P. Milet-Pinheiro. 2019. Pollination ecology of the Neotropical gesneriad *Gloxinia perennis*: chemical composition and temporal fluctuation of floral perfume. *Plant Biology* 21: 723–731.

Martén-Rodríguez, S., A. Almarales-Castro, and C. B. Fenster. 2009. Evaluation of pollination syndromes in Antillean Gesneriaceae: evidence for bat, hummingbird and generalized flowers. *Journal of Ecology* 97: 348–359.

Matallana, G., M. A. S. Godinho, F. A. G. Guilherme, M. Belisario, T. S. Coser, and T. Wendt. 2010. Breeding systems of Bromeliaceae species: evolution of selfing in the context of sympatric occurrence. *Plant Systematics and Evolution* 289: 57–65.

Mitchell, R. 1993. Adaptive significance of *Ipomopsis aggregata* nectar production: observation and experiment in the field. *Evolution* 47: 25–35.

Orr, H. A., and M. Turelli. 2001. The evolution of postzygotic isolation: accumulating Dobzhansky-Muller incompatibilities. *Evolution* 55: 1085–1094.

Pauw, A. 2006. Floral syndromes accurately predict pollination by a specialized oil-collecting bee (*Rediviva peringueyi,* Mellitidae) in a guild of South African orchids (Coryciinae). *American Journal of Botany* 93: 917–926.

Pauw, A., J. Stofberg, and R. J. Waterman. 2009. Flies and flowers in Darwin's race. *Evolution* 63: 268–279.

Perret, M., A. Chautems, R. Spichiger, M. Peixoto, and V. Savolainen. 2001. Nectar sugar composition in relation to pollination syndromes in Sinningieae (Gesneriaceae). *Annals of Botany* 87: 267–273.

Phillips, S. J., and M. Dudík. 2008. Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecogeography* 31: 161–175.

Qiu, Z.-J., Z.-L. Yuan, Z.-Y. Li, and Y.-Z. Wang. 2011. Confirmation of a natural hybrid species in *Petrocosmea* (Gesneriaceae) based on molecular and morphological evidence. *Journal of Systematics and Evolution* 49: 449–463.

Ramírez-Roa, A. 1987. Revisión de Achimenes (Gesneriaceae). Senior thesis, Universidad Nacional Autónoma de México, Ciudad de México, México.

Ramsey, J., H. D. Bradshaw, and D. W. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* 57: 1520–1534.

Rathcke, B., and E. P. Lacey. 1985. Phenological patterns of terrestrial plants. *Annual Review of Ecology and Systematics* 16: 179–214.

R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Roalson, E. H., and W. R. Roberts. 2016. Distinct processes drive diversification in different clades of Gesneriaceae. *Systematic Biology* 65: 662–684.

Roberts, W. R., and E. H. Roalson. 2018. Phylogenomic analyses reveal extensive gene flow within magic flowers (*Achimenes*). *American Journal of Botany* 105: 726–740.

Roubik, D. W., D. Yanega, M. S. Aluja, S. L. Buchmann, and D. W. Inouye. 1995. On optimal nectar foraging by some tropical bees. (Hymenoptera: Apidae). *Apidologie* 26: 197–211.

Schemske, D. W., and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences, USA* 96: 11910–11915.

Smith, J. F., J. Clark, M. Amaya-Márquez, and O. H. Marín-Gómez. 2017. Resolving incongruence: species of hybrid origin in *Columnea* (Gesneriaceae). *Molecular Phylogenetics and Evolution* 106: 228–240.

Sobel, J. M. 2014. Ecogeographic isolation and speciation in genus *Mimulus*. *American Naturalist* 184: 565–579.

Sobel, J. M., and G. F. Chen. 2014. Unification of methods for estimating the strength of reproductive isolation. *Evolution* 68: 1511–1522.

Sobel, J. M., G. F. Chen, L. R. Watt, and D. W. Schemske. 2010. The biology of speciation. *Evolution* 64: 295–315.

Tao, Z.-B., Z.-X. Ren, P. Bernhardt, H. Liang, H.-D. Li, Y.-H. Zhao, H. Wang, and D.-L. Li. 2018. Does reproductive isolation reflect segregation of color forms in *Spiranthes sinensis* (Pers.) Ames complex (Orchidaceae) in the Chinese Himalayas? *Ecology and Evolution* 8: 5455–5469.

Turelli, M., and L. C. Moyle. 2007. Asymmetric post-mating isolation: Darwin's corollary to Haldane's rule. *Genetics* 176: 1059–1088.

Whittall, J. B., and S. A. Hodges. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447: 706–710.

Widmer, A., C. Lexer, and S. Cozzolino. 2009. Evolution of reproductive isolation in plants. *Heredity* 102: 31–38.

Wiehler, H. 1983. A synopsis of the Neotropical Gesneriaceae. *Selbyana* 6: 1–219.

Zar, J. H. 2014. Biostatistical analysis, 5th ed. Pearson Education, Harlow, UK.

Zhang, X., L. Yang, and M. Kang. 2017. Post-pollination reproductive isolation of sympatric populations of *Primulina eburnea* and *P. mabaensis* (Gesneriaceae). *Biodiversity Science* 25: 615–620.

CAPÍTULO II. EVOLUCIÓN DE LOS CARACTERES FLORALES EN EL GÉNERO *ACHIMENES*.

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RESUMEN

La evolución de los síndromes de polinización frecuentemente se ha evaluado mediante la reconstrucción de estados ancestrales de caracteres florales individuales con base en una filogenia, al tiempo que se infieren los principales sistemas de polinización, y mediante la evaluación de la asociación entre las transiciones en los rasgos florales y los sistemas de polinización determinados en el campo. Además, incluso si los patrones de síndromes de polinización probablemente se deban a la selección correlacional mediada por polinizadores, aún se entiende menos si los rasgos florales están correlacionados, y si esas correlaciones describen síndromes de polinización particulares. Durante cinco años, registramos los polinizadores y la morfometría floral de 21 de las 25 especies de *Achimenes* y reconstruimos la filogenia del género utilizando tres marcadores moleculares nucleares y dos de cloroplastos. Determinamos que los fenotipos florales de las especies de *Achimenes* se dividen en cuatro grupos asociados con cuatro sistemas de polinización diferentes: polinización de abejas grandes, abejas pequeñas, mariposas y colibríes. Además, seguimos un método para describir la integración de la corola por Benítez-Vieyra *et al.*, 2019. Los cambios en el color principal y la forma de la corola ocurren simultáneamente con los cambios en los sistemas de polinización. Las medias de los caracteres cuantitativos (representados como Componentes principales) también están asociados con los polinizadores, pero los patrones de covarianza entre los caracteres solo están asociados con los sistemas de polinización de abejas grandes y pequeñas. La integración floral definida como la correlación de caracteres florales, se observó en términos de las asociaciones entre el ancho de la corola con la longitud y el ancho frontales, pero no con la longitud de la corola. Los caracteres morfológicos se ajustan mejor al modelo de Ornstein-Uhlenbeck, lo

que sugiere, evolución adaptativa y corrobora que los cambios en los sistemas de polinización están asociados con cambios en los caracteres florales. En general, encontramos que los sistemas de polinización representan una presión selectiva importante que promueve las asociaciones entre caracteres florales, que subyacen las definiciones del síndrome de polinización en las especies de *Achimenes*, pero la integración de la corola no difiere entre los sistemas de polinización, lo que indica posibles rutas de desarrollo conservadas.

Abstract: The evolution of pollination syndromes has been frequently evaluated by reconstructing ancestral states of individual floral traits in a phylogeny while mostly inferring pollination systems, and by evaluating the association between transitions in floral traits and of pollination systems determined in the field. Furthermore, even if patterns of pollination syndromes are probably due to pollinator mediated correlational selection, whether floral traits are correlated while describing particular pollination syndromes is still less understood. Over five years, we recorded the pollinators and floral morphometrics of 21 out of 25 *Achimenes* species and reconstructed the phylogeny of the genus using three nuclear and two chloroplast molecular markers. We determined that floral phenotypes of *Achimenes* species fall into four groups associated with four different pollination systems: large-bee, small-bee, butterfly, and hummingbird-pollination, and we also followed a method for describing corolla integration by Benitez-Vieyra *et al.*, 2019. Changes in corolla main color and shape occur simultaneously with changes in pollination systems. The means of quantitative traits (depicted as Principal Components) are also associated with pollinators, but patterns of covariance among traits are only associated with pollination by large and small bees. Floral integration defined as correlated floral traits was observed in terms of associations of corolla width with frontal length, and width but not with corolla length. Morphological traits better fitted Ornstein-Uhlenbeck model, suggesting adaptive evolution and corroborating that changes in pollination systems are associated with changes in floral traits. Overall, we found that pollination systems are an important selective pressure that promotes floral trait associations behind the pollination syndrome definitions in *Achimenes* species, but that corolla integration did not differed among pollination systems, indicating possible developmental conserved paths.

Keywords: floral-evolution, floral-integration, pollination-systems, pollination-syndromes

Introduction

Floral evolution is intimately related to biotic pollination (Dodd *et al.*, 1999; Kay & Sargent, 2009). The diversity of floral morphologies, attractants, and reproductive systems are often an outcome of plant-pollinator interactions (e.g. Whittall & Hodges, 2007; Schiestl & Johnson, 2013; Abrahamczyk *et al.*, 2017), where the most frequent and efficient pollinators are considered principal agents of selection on floral traits (Stebbins, 1970; Rosas-Guerrero *et al.*, 2014; Baranzelli *et al.*, 2020). Floral characters relevant to pollination interactions include morphological characters that play a role in attraction and/or pollen transfer efficiency (e.g. corolla size and shape, size and placement of floral reproductive organs), reward and attraction traits (e.g. nectar quantity and sugar content, flower color, scents, etc.), and breeding system traits. These traits are thought to have evolved partly in response to the sensorial capabilities, mechanical fit, foraging behaviour, and also nutritional demands of their animal pollinators (e.g. Martínez del Río, 1990; De Luca & Vallejo-Marín, 2013; Schiestl & Johnson, 2013; Shrestha *et al.*, 2013; Newman *et al.*, 2015; Bukovac *et al.*, 2017); however, developmental factors, pleiotropic effects, and selection by abiotic or non-pollinating agents also play a role in the evolution of floral traits (Ashman & Majetic, 2006; Strauss & Whitall, 2006; Smith, 2016).

The importance of pollinators as agents of selection on floral traits is reflected on the ubiquitous patterns of convergent floral evolution evident across a large number of plant lineages (i.e. pollination syndromes; e.g. *Ruellia*, Tripp & Manos, 2010; Gesneriinae, Martén-Rodríguez *et al.*, 2010; Mirbelieae and Bossieaeeae, Toon *et al.*, 2014; Loasoideae, Strelin *et al.*, 2015; *Gladiolus*, Valente *et al.*, 2015; Lobelioideae, Lagomarsino *et al.*, 2017). Many studies of pollination syndrome evolution depict directionality in transitions from one pollination syndrome to another. For example, in the genera *Aquilegia*, *Penstemon*, *Costus*, *Schizanthus* and *Mimulus*, there were more changes from bee to hummingbird syndromes than the reverse ones (Beardsley *et al.*, 2003; Kay *et al.*, 2005; Pérez *et al.*, 2006; Whittall & Hodges, 2007; Wilson *et al.*, 2007). However, in other genera such as *Ruellia*, transitions from hummingbird to bee and insect pollination systems were more frequent than the reverse, followed by changes from hummingbird to moth or bat-pollination systems (Tripp & Manos, 2009). In the Caribbean islands Gesneriaceae, changes from hummingbird to generalized pollination systems were due to both the unpredictability of pollination and low pollinator visitation (Martén-Rodríguez *et al.*, 2010). However, in order to understand the patterns of pollination syndrome transitions in relation to pollinator shifts, it is critical to have adequate pollination data, different floral traits describing pollination syndromes and robust phylogenies (Armbruster, 2014).

The study of the patterns of floral trait evolution under a phylogenetic context is important, because it allows to determine the number of transitions, associations with other traits, directionality of changes, etc; therefore, this approach ultimately allows us to evaluate the mechanisms associated with floral diversification (e.g. pollinator mediated selection, antagonist mediated selection, genetic drift, hybridization) (Losos, 2011). Since factors other than pollinator-mediated selection may promote changes in floral traits (e.g. herbivory, Parachnowitsch & Caruso, 2008; climate, Serrano-Serrano *et al.,* 2015; microclimate, Koski & Ashman, 2015), it is important to statistically test for associations between traits and measures of pollinator importance in order to determine the importance of pollinator as potential mediators of change in floral trait transitions (Reynolds & Fenster, 2008; Smith *et al.*, 2008; Smith, 2010; Baranzelli *et al.*, 2020). Although there is an increasing number of authors that report the statistical correlation among floral traits and pollination systems within a phylogenetic context, there are still a few of them (e.g.Smith *et al.*, 2008; Martén-Rodríguez *et al.*, 2010; Landis *et al.*, 2018; Benitez-Vieyra *et al.*, 2019). Overall, the results of most studies that evaluated floral trait correlation and pollinator systems indicate that pollinators usually account for variation in floral traits, such as flower shape and nectar volume, but flower color may variate due to other factors, rather than pollinators.

The pollination syndrome concept, is defined as a combination of floral characters that attract and reward particular pollinators (Fenster, *et al.*, 2004). Furthermore, particular combinations of floral traits that are thought to be related to pollinator adaptation are associated with higher diversification rates (O'Meara, *et al.*, 2016). Within a population framework, it has been found that some floral trait combinations affect pollinator visitation. For example in *Silene virginica*, it has been shown that hummingbirds select for floral trait combinations that include the particular height above the ground at which at which flowers are produced, red corollas and the particular horizontally held flowers, although depending on the combination, wide corollas and white color could also be favored (Fenster *et al.*, 2015). Nonetheless, whether trait correlations are themselves selected for remains less understood (Young & Badyaev, 2006).

The idea that selection acts on correlation among phenotypic traits supports the notion that phenotypic integration is a source of variation in morphological evolution (independent on the source of phenotypic integration, such as functional, genetical, developmental bases) (Armbruster *et al.*, 2014, Goswami *et al*., 2014). In plants, the correlation among floral traits is frequently interpreted as a result of either a functional process (reproductive) or of a developmental process (Esteve-Altava, 2017). It has been proposed that specific correlations among floral traits are necessary to accomplish reproduction in many angiosperm species since the precise transfer of pollen between anthers and stigmas occurs through the interaction with pollinators' mouth parts. Thus, changes in pollinators or due to environmental variation would affect fitness by altering stamen-pistil correlations and possibly other floral traits (Berg, 1959, 1960). If phenotypic integration is prone to selection, as in the case of floral integration, it could either promote or constrain changes to certain adaptive landscapes (Felice, 2018). In the case of angiosperm evolution, if particular floral correlations define pollination syndromes, then some transitions among pollination syndromes could occur more frequently than others (Stebbins, 1970).

The view that floral integration (depicted as correlation among floral traits) is the result of selection exerted by the most frequent and efficient pollinators has been documented in several studies at the population level (e.g. Reynolds *et al.*, 2009; Baranzelli *et al.*, 2014; Pérez-Barrales *et al.*, 2014; Lázaro & Santamaría, 2015) and in a small number of comparative studies that have evaluated floral integration under different hypotheses, such as the comparison of self-compatible and self-incompatible species, or the comparison of generalized and specialized species (Anderson & Busch, 2006; Pérez *et al.*, 2007; Rosas-Guerrero *et al.*, 2011; Gómez *et al.*, 2014; Joly *et al.*, 2018). However, there is limited information on the patterns of floral trait correlations among different specialized pollination systems (e.g. Bignoniaceae, Alcántara *et al.*, 2013; *Salvia*; Benitez-Vieyra *et al.*, 2019; *Erica* species, Reich *et al.*, 2020; Merianieae, Dellinger, *et al.*, 2020). Since foraging behavior and floral manipulation of pollinators imply a direct interaction with floral traits, it might be expected that species pollinated by different animals will show different patterns of correlations among floral characters (Armbruster, *et al.*, 2009). For instance, in the genus *Salvia*, floral traits describing morphology in bee-pollinated species differ from those in hummingbird-pollinated species, and there is more convergence in the morphological covariances among bee-pollinated species (Benitez-Vieyra *et al.*, 2014, 2019). Since bees have to manipulate the flowers through the staminal lever mechanism to obtain access to rewards in *Salvia*, while hummingbirds just enter their beaks into the flower, it was proposed that bee-pollinated species would be more integrated than hummingbird pollinated ones (Benitez-Vieyra *et al.*, 2014, 2019).

Alternatively, flowers might be more integrated in species highly dependent on pollinators, where floral visitation is low and flowers require high precision pollen transfer, such as in flowers with narrow tubular corollas (Fenster *et al.*, 2015). High dependence on pollinators would be present mainly in outcrossing species with a large number of ovules, which require several visits for successful pollination of all ovules. For example, in the family Gesneriaceae, both island and mainland species show low levels of pollinator visitation and have many ovules, implying that each pollinator visit is important (e.g. Martén-Rodríguez et al., 2009; Ramírez-Aguirre *et al.*, 2016, 2018). Accordingly, in two species of *Drymonia*, Ramírez-Aguirre *et al.*, 2016, found that *Drymonia strigosa*, a hummingbird pollinated species, showed greater and more statistical correlations among floral traits than *Drymonia oinochrophylla*, a large bee pollinated species. Thus, patterns of floral integration may differ among taxa.

Berg (1960) proposed that plants that were less dependent on animal pollinators would have lower floral trait correlations than plants dependent on pollinators with respect to vegetative parts, suggesting that pollinator mediated selection may affect floral trait correlations. Under the idea that more dependent pollinators would promote higher floral integration, both Pérez *et al.* (2007) and Rosas-Guerrero *et al.* (2010), found that self-compatible species of *Schizanthus* and *Ipomoea*, respectively, are more integrated than self-compatible ones, challenging the idea that pollinator dependence could promote higher integration. However, this comparison is not always possible, because many lineages are either only self-compatible or only outcrossing. For self-compatible species, autonomous pollination has been interpreted as a reproductive assurance mechanism when pollinators are scarce in pollinator-specialized plants (Fenster & Martén-Rodriguez, 2007). Although, the association between floral traits and levels of autonomous pollination has not been explored, our expectation is that, the more autonomous the plant, the less integrated it would be.

We studied the evolution of floral phenotypes in the Neotropical genus *Achimenes* (Gesneriaceae) that exhibits high floral diversity in its 25 described species. *Achimenes* is an excellent system to explore questions concerning the correlates of floral integration and floral evolution. All species of the genus occur in Mexico and species often coexist in sympatry with other congeners. Besides, all species are self-compatible but they are capable of autonomous pollination. We evaluated the evolution of ten floral traits including color, morphology and nectar rewards, in relation to pollination system transitions within a phylogenetic context. Specifically, we evaluated: (a) the association between pollination system and individual floral trait transitions (individual traits and collective suites of traits including color, morphology, and nectar rewards), (b) the patterns of floral trait correlation and covariation in association with pollination systems, (c) the association between floral trait correlations and plant breeding system. We worked under three main hypotheses. First, because different pollinators have different body morphologies and behaviours in their interaction with flowers, changes in floral shape, size, color and reward will correspond to particular pollination systems. Second, since floral traits should evolve collectively in response to pollinator-mediated selection, correlation patterns among floral traits will be different depending on pollinator systems. Specifically, we evaluated the hypothesis that bee-pollinated species had more integrated corollas than species with other pollination systems as has been found in other systems (e.g. *Salvia*, Benitez-Vieyra *et al.*, 2019). Third, following reasoning of more integration in pollinator-dependent species (Armbruster *et al.*, 1999), we hypothesize that integration would be higher in less autonomous species than in more pollinator-dependent self-compatible species. Here we assess the evolution of floral traits, floral integration and pollination systems in *Achimenes* genus, based on field observations of at least 18 species and a five-marker molecular phylogeny that encompases 23 of the 25 described species.

Materials and Methods

Study group

The genus *Achimenes* encompasses 25 accepted species (Table 1), which are all found in Mexico, although a few species occur as far south as Colombia (Ramírez-Roa, 1987; Wiehler, 1992; Ramírez-Roa & Skog, 2002; Beutelspacher & Martínez-Meléndez, 2008). *Achimenes* species inhabit shaded, humid and rocky microhabitats across different vegetation types through Mexico, primarily in seasonal habitats. Some species are found in sympatry occupying particular wet microhabitats of seasonal environments, and pollinator specialization is the most important reproductive isolation mechanism in some of these species assemblages (Ramírez-Aguirre *et al.*, 2019).

Achimenes species show five main corolla colors (white, yellow, purple, pink, red) and three corolla shapes (infundibiliform, hypocrateriform, and tubular) (Ramírez-Roa, 1987). *Achimenes* species are all protandrous (the staminate phase develops first) and they are mostly out-crossing but all are self-compatible (Martén-Rodríguez unpublished data). In the field anthesis lasts 4-5 days, while in the greenhouse anthesis could last 5-6 days in some species, such as *A. patens*, but is not a generalized pattern. A previous phylogeny was proposed with two molecular markers for 20 species (Roalson *et al.*, 2003), and a more recent phylogeny based on transcriptome assemblies was proposed for 10 species (Roberts & Roalson, 2018).

Plant collection and sampling design

Species distribution ranges were obtained from TROPICOS or GBIF data-bases and from the following herbaria: MEXU, XAL, SERO, IEB, IBUG, HEM, and on-line ARIZ. Based on this information we collected plant samples along five mountain ranges in Mexico between sea level and 2400 m.a.s.l.: a) Sierra Madre Occidental, b) Trans-Mexican volcanic belt, c) Sierra Madre del Sur, d) Sierra de Chiapas, and e) Sierra Madre Oriental. Specimens were collected on hillsides along roads, waterfalls and river canyons, which encompass the main habitats for *Achimenes* species (shaded rocky substrates cliffs and river banks). We sequenced five molecular markers for 22 *Achimenes* species and three outgroup species from the subtribe Gloxiniinae (Weber *et al.*, 2013), including putative sister genera *Smithiantha*, *Eucodonia* and *Moussonia* (Table 2). We established a greenhouse collection of 23 *Achimenes* species at Universidad Nacional Autónoma de México, campus Morelia.

DNA extraction and amplification protocols

Molecular work was conducted in the National Laboratory of Ecological Analysis and Synthesis (LANASE, ENES-UNAM, Morelia, Campus). DNA was extracted from dried leaf material and from the above mentioned greenhouse collection of *Achimenes*. We followed a modified CTAB DNA extraction protocol from Doyle & Doyle (1987; see Supporting Information 1 for complete description). Single marker amplifications were conducted for all species using Multiplex PCR Kit (Qiagen, Germany). We used three nuclear (ITS, ETS, GCYC) and two chloroplast regions (rpl32-trnL, trnL-trnF). Amplification protocols including primer temperatures for all molecular markers followed manufacturer's instructions (Supporting Information Table S1). All samples were sequenced at Macrogen Inc. at South Korea with the company's standard purification and standard sequencing protocols.

Pollination system description

Pollinators were recorded directly and with video cameras. Observations for all species were conducted during the day between 8:00 and 16:00 hrs. Nocturnal observations were conducted for a few species only because most study sites were not safe at night. For species whose floral traits indicated nocturnal pollination might be important (*A. longiflora*) we conducted observations between 18:00 and 22:00 hrs and between 5:00 and 9:00 hrs. The number of observation hours ranged from 6 to 25 across species. Floral visitors were considered pollinators when they introduced beaks, proboscis or heads into flowers contacting the reproductive organs (Table 1).

Floral traits and pollination syndromes

In order to study the evolution of floral traits and floral integration in *Achimenes* we used traits associated with morphology, nectar rewards and color. To describe floral morphology, we measured nine traits that describe flower shape: 1) corolla length, 2) corolla width, 3) corolla length at mouth, 4) corolla width at mouth, 5) lower petal length, 6) frontal length, 7) frontal width, 8) corolla base (Figure S1). We measured one flower per individual of 15 to 30 individuals of each species, according to population sizes and number of available individuals. Measurements were taken with an analog caliper to 0.01 mm precision (Monticello, MN, US). Floral shape for comparative analyses used shape categories based on Ramírez-Roa (1987) as follows: (a) infundibiliform, describes flowers with corollas wider than their length, similar to a bell; (b) hypocrateriform, describes flowers with corolla tubes narrower than their length and with extended corolla lobes, i.e. narrow long tubes with a flare; (c) tubular describes flowers with relatively cylindrical flowers with little or no flare. The floral traits of *Moussonia deppeana* outgroup were obtained from Ramírez-Aguirre (2011).

We sampled nectar volume from plants located in the greenhouse. We tagged at least three haphazardly chosen flowers from one to ten individuals of each species. Nectar was collected from flowers on the first day of female anthesis, using Drummond Microcaps (Broomall, PA, US) 1 µl and 5 µl. We did not use measures of nectar sugar concentration (°Brix) because our previous measures did not suggest differences among species (Ramírez-Aguirre *et al.*, 2019, and Martén-Rodríguez *et al*. unpubl).

Corolla color was defined by visual similarity with https://colorpalettes.net/ palette colors using flowers from greenhouse plant specimens during early morning hours (Supporting

Information Table S2). We chose mornings because light was most available, and corolla color does not change during anthesis, so at any time flowers would have the same color.

Assessment of floral integration index

To evaluate floral shape in multivariate space we conducted a phylogenetic Principal Component Analysis (PCA) on natural logarithm-transformed floral measurements (statistical means) to evaluate if different floral phenotypes are associated with particular pollination systems. We also estimated floral integration index following equation in Pavlicev *et al.* (2009), which is based on the variance of the eigenvalues of a correlation matrix, corrected for number of observations. Integration indexes were calculated with the PHENIX package in R program (Torices & Muñoz-Pajares, 2015).

A phylogenetic PCA was performed once we obtained our five marker phylogeny to understand floral syndrome definitions in *Achimenes* genus with eight morphological characters alone (21 *Achimenes* species) and two outgroups. A phylogenetic generalized least squares (PGLS) was performed to understand association between observed main pollinators and morphospace defined by PCA. These analyses were done with natural logarithm-transformed means of floral measurements. Results from these analyses are described in Supporting Information Figure S2 and in Table S4.

Autonomous self-pollination and floral integration relation.

We measured autonomous pollination in *Achimenes* species at the greenhouse since reproductive systems affect floral integration (e.g. Rosas-Guerrero *et al.*, 2010). Flowers of 18 *Achimenes* species were marked and followed through fruit development in the greenhouse to evaluate potential for autonomous pollination (Martén-Rodríguez unpubl.).

Five to 20 flowers per individual were marked for each species, depending on plant size. Mean fruit-set due to autonomous pollination was obtained for 18 species. However, since there is low variation in autonomous pollination, we evaluated the association between floral integration indexes and potential autonomous pollination with a Kendall test, due to violations to PGLS assumptions (Mundry, 2014).

Phylogenetic reconstruction

Sequences were edited with Sequencher software version 5.4.5., and aligned with MAFFT algorithm with E-INS-i iterative refining method for sequences with multiple conserved domains and long gaps (Katoh *et al.*, 2017). GCYC sequences were also aligned with MAFFT, but with G-INS-I with iterative refinement for sequences with an accurate guide tree (Katoh *et al.*, 2017). Alignments were manually edited in PhyDE® program, version v0.9971 (Müller *et al.*, 2010) and a concatenated matrix of the five molecular markers was assembled in Mesquite version 3.6 (Maddison & Maddison, 2018). Statistics of individual marker alignments was done in MEGA version X program (Kumar *et al.***,** 2018) and are described in Supporting Information Table S3.

We first obtained nucleotide substitution models for individual markers with JModelTest (Darriba *et al.*, 2012). For phylogenetic analyses we used Bayesian inference with the concatenated matrix of ITS, ETS, GCYC, rpl32-trnL and trnL-trnF markers in MrBayes program (Ronquist *et al.*, 2011). We used a GTR model with a gamma distribution for ITS, ETS, rpl32-trnL and trnL-trnF, and a GTR with “equal” distribution model for GCYC. We ran the analysis for 10 000 000 generations with a 50% burnin, a 1000 sample frequency and four separate chains. Analyses with JModelTest, MrBayes were performed on the CIPRES portal (Miller *et al.*, 2010). To evaluate chain convergence and to obtain other statistical descriptors of the MCMC of MrBayes analyses, we used the *RWTY* package (Warren *et al.*, 2019) in R program (RCore Team, 2019).

The total evidence consensus tree was first rooted with three outgroups, then the three polytomies on the tree were solved with "multi2di" function with "random parameter=FALSE" of *ape* package (Paradis & Schliep, 2019). Also, whenever there was missing data either for a species (for example, *A. ixtapaensis*) or for a population of a species (for example, *A. grandiflora2*), these taxa were pruned from the tree and phylogenetic analyses were performed with the complete data set. Since polytomies had zero branch lengths, they were re-calculated with "compute.brlen" function with Grafen's method of ape package (Paradis & Schliep, 2019; Grafen, 1989). This method re-calculates branch lengths as the difference in height of re-scaled lengths to one (the root). With this branch length transformation zero branch lengths are avoided and this also made the tree ultrametric.

Floral trait evolution

For analyses of discrete characters, such as corolla shape, colour and observed pollinators, we followed a Bayesian approach. We used the BayesTraits v.3 program with Reversible Jump Markov Chain Monte Carlo (RJ MCMC) with the trees generated from posterior distribution of the MCMC from MrBayes (Pagel & Meade, 2006). Ancestral reconstruction of discrete characters was done with the function MULTISTATE, and MRCA (Most Common Recent Ancestor) that allows for reconstruction of ancestral states with node supporting posterior values less than 100% (Pagel & Meade, 2006). We used the Reversible Jump method with a Hyper Prior and an exponential distribution with range values of 0 to 30 and 10 000 000 iterations. To estimate the marginal likelihood of the model, a Stepping stone analysis was run. We used the "Stones" command with 100 stones and 100 000 iterations. We evaluated ancestral states in 22 nodes selected from the Bayesian consensus tree, and used the "AddMRCA" command to indicate those nodes for the ancestral state reconstruction. The MRCA reconstruction was evaluated in the 7544 trees from the posterior distribution after the burnin step of the MrBayes analysis for both separate runs. Upon completing the two separate runs, we merged chains and analyzed convergence and statistics with *coda* package in R program (Plummer *et al.*, 2006; R Core Team, 2019). Models of evolution of discrete characters (pollinator, shape and color) were conducted with "fitDiscrete" function of the *geiger* package in R (Revell, 2012; R Core Team 2019) and the models Akaike Information Criteria (AIC) scores are on Supporting Information, Table S5.

Models of floral evolution were fitted with "fitContinuous" function of the *geiger* package (Harmon *et al.***,** 2008) with log converted values of corolla width and corolla length separately. Model selection was based in corrected Akaike Information Criterion (AICc) for Brownian Motion (BM), Early Bust (EB), Ornstein-Uhlenbeck (OU), lambda, kappa, drift and white-noise models. Also, since floral trait evolution may be explained by directional or stabilizing selection due to pollinators, we followed Butler and King's (2004) approach for testing OU models in *ouch* package in R program (King & Butler, 2019). For King and Butler's approach, it is necessary to propose selective regimes on branches in order to model OU evolution of phenotypic traits, so we used *BayesTraits* ancestral state reconstruction on the 22 nodes of the rooted and ultrametric consensus tree from MrBayes to formulate an adaptive hypothesis on continuous floral trait evolution. The other proposed painted regime was a "small bee", this model implies that there is a single regime affecting the trait, and we chose it because it was the ancestral state of the clade, after BayesTraits reconstruction. Two additional models, the Brownian model and “global” model were tested as in King and Buttler’s (2004). Through phylogenetic generalized least squares (PGLS) we evaluated the correlation between corolla color and shape and observed pollination systems with the *ape* package in R (Paradis & Schliep, 2019; R Core Team, 2019).

Floral integration evolution

We tested for correlation among pollinator, corolla shape and corolla color with“fitPagel” function of the *phytools* package with binarized character states and the pruned consensus tree (see “Phylogenetic reconstruction” section, Revell, 2012; R Core Team 2019). This function is based on Pagel’s (1994) Discrete-dependent and Discrete-independent models. Specifically, we tested hummingbird-tubular, hummingbird-red, red-tubular, butterfly-hypocrateriform, butterfly-purple, purple-hypocrateriform, bee-infundibiliform, bees-white, white-infundibiliform associations. We report, likelihood ratio test (LRT) of model selection.

For continuous data, we performed a phylogenetic least squares analysis (PGLS) to identify the association between corolla width as the dependent variable, and frontal width and length and corolla length as independent variables with *ape* and *nlme* packages with a Brownian motion and a Ornstein-Uhlenbeck correlation structures in R program (Paradis & Schliep, 2019 Pinheiro *et al.*, 2019; R Core Team, 2019). A PGLS between log transformed corolla length and nectar volume was conducted with *ape* and *nlme* packages as tested in Ornelas *et al.* (2007). A Multivariate Analysis of Variance (MANOVA) was used to test if observed pollinators predicted patterns of covariance among floral traits with corolla length and width, petal length, entrance width and frontal length (traits with the highest interspecific variability). This analysis is based on a variance-covariance matrix generated under a Brownian motion model and was conducted with *geiger* package in R program (Harmon *et al*., 2008). All tests were performed with log-transformed variables. We also report phylogenetic signals of log-transformed individual variables in Table S7.

We performed an ancestral reconstruction of integration indices with “fastAnc” function (with ML estimation) in *phytools* package (Revell, 2012) to test for changes in integration indices among pollination systems. We show reconstructed integration index values on phylogeny in Supporting Information Figure S4.

We tested for BM and OU models of evolution with different extensions, such as BM1 (Brownian motion with one rate), BMM (Brownian motion with multiple rates), OU1 (Ornstein-Uhlenbeck with one optimum), and OUM (Ornstein-Uhlenbeck with multiple adaptive optima) to understand the evolution of flowers as complex phenotypes. We used corolla width and length, entrance width, frontal width, and petal length to test for multivariate evolution models. Floral traits were log-transformed before analyses. Model selection was based on AICc values.

Comparison among covariance matrices and their relation with pollinators

We followed the approach from Benitez-Vieyra *et al.* (2019) to evaluate differences among the variance-covariance matrices of floral traits for 21 *Achimenes* species. We explored how differences in covariance structure (phenotypic integration) of continuous traits are related to different specialized pollination systems. Corolla length, corolla width, entrance width, petal length, and frontal length were used for individual species covariance calculations. To measure differences among all covariance matrices, a Riemann distance calculation was done with “MatrixDistance” function of the *evolqg* package (Melo *et al.*, 2015), generating a one-distance matrix with all species. With this new distance matrix, a Principal Coordinates Analysis (PCoA) was conducted (Benitez-Vieyra *et al.*, 2019). To understand how species covariances were distributed in space according to pollination we explored the Pearson correlation between the distance matrix and each PCoA axis. Three PGLS were run with each of the first three principal coordinates of the previous PCoA and the main pollinator system to understand how covariance structure changes with pollinators. In addition, a PERMANOVA with the distance matrix generated from covariance matrices of species.and a phylogenetic ANOVA with the three axes generated from PCoA. These tests were performed to test for differences among pollination system groups.

Apart from considering covariances, we followed Benitez-Vieyra *et al.* (2019) comparison between mean morphospace defined by phylogenetic principal component analysis with log transformed mean floral traits (in this case, with the same five traits considered for covariance matrices) with covariance morphospace. This comparison is based on Mantel tests, which tests for partial correlation among matrices. By this mean we could understand how patterns of covariation describe floral integration in *Achimenes.* Multivariate phylogenetic signal tests were performed for these five floral traits with “K.mult2 function of *phylocurve* package in R. In addition, a PERMANOVA with the Euclidean distances generated from PCA scores, and a phylogenetic ANOVA with the log transformed mean morphological traits. These tests were performed to test for differences among pollination system groups.

Results

Phylogenetic reconstruction

The total evidence phylogeny supported *Achimenes* species as a monophyletic group, with two main clades supported by a posterior probability (PP) > 0.999, representing a clade comprised by species with southern distribution and a clade of species that are distributed across Mexico (Figure 1). Most relationships are well resolved, except for a group within the latter clade, which has a posterior probability value below 0.8. Tree topology mostly corresponds to that reported in the 19 species ITS and trnL-trnF phylogeny by Roalson *et al*. (2003) and the 10 species transcriptome phylogeny by Roberts & Roalson (2018), except for the position of *A. candida, A. antirrhina,* and *A. patens* species.

Observed pollinators

Most *Achimenes* species have highly specialized pollination systems at the local or regional level, whereby, most species were pollinated by one to three animal species**.** Overall, the pollination systems of *Achimenes* species include small bees (Halictidae and Apidae-Meliponini), medium-large bees (Anthophoridae), hummingbirds, and lepidopterans. Three species are visited by two different pollinator groups (e.g. *A. erecta, A. grandiflora, A. cettoana*; Table 1). Three species were visited by pollinators that did not completely correspond with pollination syndromes *(A. admirabilis* and *A. erecta* were visited by small bees and butterflies, respectively, instead of hummingbirds; *A. occidentalis* was visited by butterflies instead of small bees; Table 1).

Pollination syndromes

Phylogenetic PCA of morphometric floral traits, resulted in four groups of species that defined the four different pollination systems observed in the field (Supporting Information Figure S2); likewise, the most informative principal components are associated differently with pollinators (Supporting Information Table S4). Pollination syndrome definition was based on commonalities found within flowers in PCA and based on pollinator observations. We defined four pollination syndromes: small bee, large bee, hummingbird and butterfly. Small bee flowers are white and infundibiliform and <30 mm corolla length, large bee flowers are white or purple and infundibiliform and ≧30 mm corolla length. Hummingbird flowers have red or orange-red tubular corollas and ≧30 mm corolla length. Butterfly flowers are purple, red or white and hypocrateriform with >20 mm corolla length at superior the petal. Nectar volume tended to be higher in hummingbird pollinated species than in any other syndrome (Supporting Information Figure S3).

Floral trait evolution

Model comparison for pollination systems, corolla color and corolla shape, resulted in better support (smaller values of AICc) for ER models for all three discrete traits (Supporting Information Table S5). For continuous individual traits (corolla length and corolla width), models of evolution suggested that Ornstein-Uhlenbeck model better described data, suggesting that selection by pollinators may be pulling traits towards an optimum (Supporting Information Table S6).

Pollination by small-bees was the ancestral condition in the genus *Achimenes* and pollination system transitions were observed in six nodes. Considering the most likely ancestral state, there were three transitions from small bee to large bee, two to butterfly, and one ambiguous node suggesting a transition from small bee to hummingbird or butterfly. There were also two transitions from large bee to hummingbird and one to butterfly. Finally, there were two transitions from butterfly to small bee (Figure 2). There were no transitions from hummingbird to any other pollination system, and no transitions from butterfly to hummingbird or large bee. The highest transition rates in pollination systems include small bee to the three other pollination systems, and large bee to hummingbird (Figure 2).

Corolla color was a five state trait, which generally followed the same trend as the pollination system reconstruction. White color was the ancestral state in *Achimenes* clade (Figure 4). Considering the most likely ancestral state for each node, transitions occurred from white to purple (3), red (2), and yellow (1), from red to purple (2) and white (1), and from purple to red (2). Mean transitions were the highest from white to any other color, while the lowest transition rates were from yellow and pink from any other color. When circumscribing to the three main colors, white, purple and red, mean transitions from white to purple, white to red where higher than the reverse. Mean transitions of purple-red, red-purple and red-white were similar (Figure 3).

The ancestral state for corolla shape was infundibiliform, from which hypocrateriform (5) and tubular (2) corollas were derived. There was one change from hypocrateriform to infundibuliform and one to tubular. There were no transitions from tubular corollas to any other shape (Figure 4).

Floral integration evolution

Discrete character correlations better fitted the dependent model in hummingbird-tubular (LRT = 18.091, df = 4, P = 0.001), hummingbird-red (LRT = 10.831, df = 4, P = 0.028), tubular-red (LRT = 15.677, df = 4, P = 0.003), butterfly-hypocrateriform (LRT = 17.401, df = 4, P = 0.002), bees-infundibiliform (LRT = 14.104, df = 4, P = 0.007). In contrast, butterfly-purple (LRT = 4.736, df = 4, P = 0.315), hypocrateriform-purple (LRT = 3.136, df = 4, P = 0.535), bees-white (LRT = 6.560, df = 4, P = 0.161), and white-infundibiliform (LRT = 4.568, df = 4, P = 0.334) better fitted the independent model.

Also, PGLS associations among floral traits indicated that corolla width was significantly associated with frontal width (t = 4.069, P= 0.0007) and length (t = -3.335, P = 0.0035) but not with corolla length (t = 0.835, P= 0.414) with a Brownian correlation structure (AIC of the model 26.99, and Blomberg's K phylogenetic signal of residuals = 0.32). The same model with an Ornstein-Uhlenbeck correlation structure showed a similar pattern, corolla width was significantly associated with frontal width (t = 4.096, P= 0.0006) and length (t = -3.390, P = 0.0031) but not with corolla length (t = 0.952, P= 0.353), but with an AIC = 23.71 and a phylogenetic signal of residuals of K = 0.31. The association between nectar volume and corolla length was statistically significant but with no phylogenetic signal (t = 3.825, P = 0.0014, Blomberg's K = 0.13). The multivariate model of evolution suggested that the OU model with one optima better described evolutionary changes in individual floral traits (AICc = 148.17, 35 parameters) (Table S8).

Floral integration indexes varied among species, but without statistical differences in floral syndromes (X^2 = 0.69, df = 3, P = 0.88; Figure 5 and Figure S4). In contrast, multivariate associations between floral traits and pollinators were significant under a phylogenetic MANOVA (F = 5.46, numDF = 3, denDF = 41.81, P = 0.0099), and multivariate phylogenetic signal for mean values of floral traits was low and no statistically significant (K_{MULT} = 0.1456, P = 0.296). As for our predictions for more integrated flowers with less autonomous pollination, we found that there was no statistical association among these two measures (tau = 0.047, P = 0.82).

Comparison among floral covariance matrices in relation to pollination systems

When considering five floral traits to calculate covariance matrix morphospace dispositions, four different groups of species were clustered by pollination system (Figure 6a). Small bee, hummingbird and butterfly-pollinated species overlapped in space in some degree, while large bee- pollinated species showed less overlapping with respect to the other pollination systems. Principal coordinates were associated with bee pollination, for example, PCo1 was significantly associated with large bee pollination (t = -2.169, P = 0.0445) and with small bee pollination (t = 3.281, P = 0.004, respectively). Principal coordinate 2, was associated with small bee pollination system (-2.744, P = 0.0138), but not with the other pollination systems (large bee, t = -0.849, P = 0.408; butterfly, t = 0.513, P = 0.614; hummingbird, t = -1.076, P = 0.297). And the third principal coordinate was not statistically related with any pollination system (large bee, t = -0.330, P = 0.746; small bee, t = -0.437, P = 0.667; butterfly, t = -0.0099, P = 0.992, hummingbird, t = -1.002, P = 0.330). PCo1 explained 57.57% of variance, PCo2 explained 12.42% and PCo3 explained 7.34% of total variance. Multivariate phylogenetic signal of principal coordinates was not significant (K = 0.141, P = 0.63). The PERMANOVA test showed not statistically significant differences among pollination systems (F = 1.567, df = 3, P = 0.144), and phylogenetic MANOVA showed marginally significant differences in at least one group (F

= 2.428, df = 3, numDF = 9, denDF = 36.657, P = 0.0519). We also found that PCo1 was mainly and negatively correlated with the frontal axis of the flower (entrance, frontal width and length, and petal length) and with corolla width. The PCo2 was negatively correlated with the entrance, corolla and petal lengths, while the PCo3 was positively correlated with corolla length and the frontal axis of the flower (frontal and petal lengths) (Figure 6b).

Principal components with the five variables used in covariance matrices constructruction were also associated with pollination systems. The first principal component (62.9% of explained variance) was associated with large bees (t = -3.166, P = 0.0056), but no with other pollination systems (small bee, t = 1.710, P = 0.1054; butterfly, t = -0.095, P = 0.9251; hummingbird, t = -1.066, P = 0.301). The second principal component (24.2% of explained variance) was associated with both large and small bees (large bee, t = -4.719, P = 0.0002; small bee, t = -6.763, P = 0.00005), but not with butterfly or hummingbird pollination systems (butterfly, t = 1.285, P = 0.216; hummingbird, t = -1.379, P = 0.1857). The third principal component (10.15% of explained variance) was associated with hummingbirds (t = 0.521, P = 0.0001), and not with the other pollination systems (large bee, t = 1.675, P = 0.1122; small bee, t = 1.583, P = 0.1318; butterfly, t = -0.577, P = 0.571). Multivariate phylogenetic signal of the five floral traits circumscribed to *Achimenes* clade resulted in not significant phylogenetic signal (K = 0.104, P = 0.201). The PERMANOVA test showed statistical differences among pollination systems (F = 10.009, df = 3, P = 0.0001) and phylogenetic MANOVA also showed significant differences in at least one group (F = 4.465, df = 3, numDF = 15, denDF = 36.289, P = 0.0009). Comparison between morphospace (with means of floral traits) and covariance space resulted in non-significant association between matrices (r = -0.057, P = 0.76).

Discussion

Evolution of floral traits and pollination systems

We found that pollination by small bees is the most likely ancestral pollination system in the genus *Achimenes* and that evolutionary transitions mostly went from small bees to other pollination systems, including large bees, butterflies and hummingbirds (Figure 2). We did not detect reversals from hummingbird to bees, but we found reversals from butterfly to small bees. Most studies involving pollination by bees and hummingbirds have found that bee pollination is generally the ancestral state and that changes from hummingbird to bee-pollination are null or rare (Wilson *et al.* 2007; Whittall & Hodges 2007; Abrahamczyk, *et al.*, 2014; Strelin, *et al.*, 2016a). A reversal from hummingbird to small bee or large bee would imply changes in corolla shape, size and color that might be developmentally or physiologically constrained (Wilson *et al.*, 2006). However, the relative young age of the genus *Achimenes* (~10 my, Roalson & Roberts, 2016) may also explain why there are no reversals in the floral traits involved in attraction and effective pollen transfer by bee pollinators, such as corolla color and shape. Regardless of the mechanism, results indicate that evolutionary transitions in pollination systems are associated with changes in floral morphology and color in *Achimenes* (Figs. 2, 3, 4).

The analysis of flower color evolution indicated that this trait is highly labile in the genus *Achimenes*. Other studies have shown that changes from white to other colors are less frequent than the opposite, due to regulatory loss or inactivation of genes involved in anthocyanin production (Rausher, 2008). However, our results are similar to those found by

Kay *et al.* (2005) in that there was a high frequency of changes from white to coloured flowers, indicating that other mechanisms are involved. *Achimenes* with white corollas have nectar guides of different colors, for example, purple, yellow, dark red spots or lines, suggesting that regulation in the distribution of anthocyanins within corollas, not complete inactivation of anthocyanin metabolism at the whole flower level, may underlie corolla color transitions. In fact, flower patterning color is related to both tissue and cell type specificity in *Petunia*, *Ipomoea* and in *Antirrhinum* (Martin and Gerats, 1993; Bradley *et al.*, 1998; Durbin *et al.*, 2003). This kind of regulation is associated with MBW transcription factors, specifically R2R3MYB, which determine the site and temporal expression of genes that produce color (Davies *et al.*, 2012; Albert *et al*., 2014). In *Aquilegia*, changes from red to blue are probably related to changes in expression rather than loss of function, as is the case of white-flowered *Aquilegia* species, which still retain capacity to produce anthocyanins in other tissues (Hodges & Derieg, 2009). This pattern is also found in *Achimene*s species, since there are reddish abaxial parts of leaves and stems in addition to red or purple nectar guides. Thus, it is possible that in *Achimenes* floral color diversity is determined by regulation at the level of gene color expression rather than complete modification of color biosynthetic pathways, allowing for diverse floral color patterns, which has been proposed for other plant groups (Durbin *et al.*, 2003; Specht & Howarth, 2015).

Another pigment source that is present in flowers are carotenoids, which are responsible for yellow colors in flowers. Carotenoids are mainly related to photoreception, photoprotection and herbivore deterrence, but they are also related to seed-disperser and pollinator attraction (Cazonelli, 2011). In *Mimulus* the *yup* locus controls for carotenoid deposition in flowers, which reduces bee visitation, and its combination with an allele that controls for higher nectar volumes increases hummingbird visitation (Schemske & Bradshaw, 1999). Furthermore, in *Mimulus lewisii*, there is a MYB transcription factor (a candidate for GUIDELESS gene), that affects carotenoid deposition on nectar guides, trichome complexity and epidermal cell development, suggesting that these pleiotropic effects could potentially also affect pollinator visitation (Yuan *et al.,* 2013). In *Achimenes* genus there is one species with mostly yellow flowers (*A. flava*), five species with yellow throats enclosed in red corollas, three with light-yellow throats enclosed in white corollas, and two species with light-yellow throats enclosed in purple corollas, suggesting that carotenoid pigments are widespread and less important in pollinator differentiation.

In terms of floral shape, flowers in *Achimenes* and many Gesneriaceae species share a basic pattern of zygomorphic corollas with fused petals, but they show high variation in corolla shape and size. This diversity may result from variation in a few loci of medium effect as in *Penstemon* (Wessinger *et al.*, 2014) or in changes in regulatory pathways that affect allometric relationships among floral traits (Zhong & Preston, 2015; Wózniak & Sicard, 2018). In either case, traits that conform floral shape are often associated with pollinator traits. In *Achimenes* there is association among corolla length and most frequent pollinators' proboscis or beaks, suggesting that pollinators mediate selection of flower shape (Ramírez-Aguirre *et al.*, 2019).

Our results also support the notion of different patterns of floral trait associations in relation to different pollination systems. Based on the pollination syndrome concept, functional associations among floral traits would be expected as a response of correlated selection mediated by different pollinator functional groups (Fenster *et al.*, 2004). We found that both covariance space (which reflects floral integration) and morphospace (which reflects mean trait associations) are grouped by the four observed functional groups of pollinators (Figure 6; Table S4). However, there were no statistical differences among covariance matrices of different pollination systems. These results suggest that in *Achimenes* different pollinators favour different trait associations, but that developmental trait associations are also involved in pollination syndromes. In either case, some groups of species were more similar than others, suggesting that different pollinators may exert similar pressures on some traits. For example, in PCA and in PCoA hummingbird-pollinated species are closer to butterfly-pollinated species (Figures 6 and S2); both groups have narrow floral tubes that would fit birds' beaks and butterflies' proboscides. Changes from hummingbird to butterfly or vice versa would require small changes in corolla length and width, although changes in volatiles may also be expected (Andersson *et al.*, 2002). One might hypothesize that, given enough time, evolutionary transitions between these floral syndromes might be more common than transitions to or from bee flowers, which would require larger changes in corolla shape, color and rewards.

Specialization in pollination systems is the rule in *Achimenes* species; however, some species had less clearly defined pollination syndromes and were visited by animals not expected by the syndrome. These species include *A. admirabilis* (hummingbird-butterfly syndrome, pollinated by small bees), *A. erecta* (hummingbird-butterfly syndrome pollinated by butterflies) and *A. occidentalis* (small bee syndrome, pollinated by butterflies). It is possible that alternative, less frequent pollinators were missed during observations and that floral traits actually reflect selection by two different pollinator functional groups; e.g., *A. erecta* with relatively short narrow floral tubes associated with butterflies (observed) and red color associated with hummingbirds (not observed). Mixed pollination syndromes have been reported in some plant species (bee-hummingbird, for example in *Penstemon*, Lara & Ornelas, 2008; *Drymonia*, Ramírez-Aguirre *et al.*, 2016), bird-bat in *Burmeistera tenuiflora* (Campanulaceae) and *Paliavana sericiflora* (Gesneriaceae), both showing traits associated with bat and hummingbird pollination (Muchhala, 2003; SanMartín-Gajardo & Sazima, 2005). In these cases, floral traits may represent intermediate or suboptimal adaptive states of transitional systems (Wilson *et al.*, 2006), or they may be associated with different selective pressures exerted by different groups of effective pollinators (Muchhala *et al.*, 2009).

In *Achimenes,* bee-pollinated species form two groups of species separated primarily by size. The "small bee" syndrome showed more variation in flower shape (infundibiliform and hypocrateriform corollas) than the "large bee" syndrome (infundibiliform corollas only), which is probably related to the foraging behavior of bees. For instance, small bees, such as *Trigona fulviventris* and Halictidae, actively collected nectar and pollen in two major ways: either they entered and exited the flower facing downwards, or they entered the flower, turned their bodies and exited facing upwards presumably to collect pollen. The latter behavior resulted in pollen deposition on the bee´s adaxial thorax, suggesting sternotribic pollination. In contrast, large bees always entered and exited flowers facing downwards while pollen was deposited on the abaxial thorax, suggesting nototribic pollination while feeding on nectar. These differences in foraging behaviour among bees, may also be related to the types of rewards offered by flowers. Wiehler (1976) and Ramírez-Roa and Skog (2002) hypothesized that *A. mexicana*, *A. dulcis*, *A. glabrata* and *A. hintoniana*, are gynandro-euglossophylous species, i.e. pollinated by both female and male

Euglossine bees. We documented mostly female Euglossine bees in the above mentioned species (except for *A. glabrata*), but males were rare visitors as well. Male euglossine bees are known to collect floral scents, which are allegedly involved in mating (Dressler, 1968). Future studies should assess scent production in relation to Euglossine bee pollination in the family Gesneriaceae.

In the case of butterfly and hummingbird pollinated species, these animals only seek for nectar while contacting reproductive floral organs in a spot with either probosces or beaks. We observed pollen deposition near the beginning of the proboscis of a butterfly on *A. grandiflora*. In the case of hummingbirds, pollen deposition should be in the foreheads or the crown of the head, as was observed in other Gesneriaceae species (Martén-Rodríguez e*t al.*, 2009). In contrast to bees, which interact with their whole body with flowers, both butterflies and hummingbirds have little contact with other body parts with flowers, which would require a bigger amount of precision to introduce their mouth parts in narrow and long corollas, hence extending handling-times and affecting nectar intake and affecting visitation (e.g. Montgomerie, 1984; Temeles, 1996). Despite differences in pollinator system flower manipulation in *Achimenes*, we did not find differences in integration patterns neither with Integration Index (Kruskall-Wallis test and Figure S4) nor by comparing covariance matrices (PERMANOVA). Interestingly, the phylogenetic MANOVA suggests possible differences among covariance patterns and when accounting for phylogenetic relatedness, suggesting that detectable differences could be found in larger taxa and that historical features mediating floral development could be involved.

Floral trait integration indices in *Achimenes* did not differ between pollination systems. A literature review across 16 angiosperm families found that integration indices tended to be generally low (around 20%) (Ordano *et al.*, 2008), which might be a consequence of the occurrence of correlated clusters or modules within flowers (intrafloral integration). Intrafloral integration would be the result of pollinator mediated selection on separate sets of floral parts. For example, floral traits involved in attraction might covary in particular ways that differ from the covariation of traits involved in effective pollen removal and deposition (e.g., Rosas Guerrero et al., 2010); however, selection on traits that conform attraction and reproduction modules varies across different research groups (e.g. Dellinger *et al.*, 2019; Reich, *et al.*, 2020). Our results coincide with Benitez-Vieyra *et al.*, 2019, in the lack of an association between covariance and morphospace matrices, suggesting that changes from one pollinator to another do not alter overall floral integration. Other factors, such as fixed and opposing ontogenetic pathways and other environmental factors, like herbivores, may favour low levels of floral integration (Fornoni *et al.*, 2008; Wessinger & Hileman, 2016). We observed very low levels of herbivory on floral structures and even in other structures in *Achimenes*, so the possibility of herbivory as a determinant of floral integration may be excluded. In a study of South American Bignoniaceae, both covariance and morphospace matrices were similar, suggesting that developmental patterns do not change between species with different pollination syndromes (Alcántara *et al.*, 2013). In *Achimenes* the role of developmental constraints in the evolution of floral trait associations is still unknown.

Armbruster *et al.*, (1999) hypothesized that floral integration would be higher in out-crossing species than in selfing species. This prediction is not actually supported by evidence as self-pollinated species tend to be more integrated than out-crossing species (e.g. Rosas-Guerrero *et al.* 2010; Fornoni *et al.*, 2015). This is probably because precise disposition of sexual organs is required to accomplish self-pollination. *Achimenes* species are self-compatible, and we expected floral integration to be associated with the level of autonomous self-pollination (AF). Nonetheless, we did not find differences in floral integration indices in relation to autonomous pollination, suggesting that higher dependence on pollinators does not affect integration. These results should be taken with caution however, as most *Achimenes* species had relatively low potential for self-pollination (lower than 5%) when floral visitors were excluded; thus, this question still requires testing in a group with higher variation in the level of autonomous pollination.

Conclusions

Specialized pollination systems in *Achimenes* probably underlie the evolution of floral traits both individually and collectively. Taken individually, corolla color and shape correspond with changes in pollination systems in the *Achimenes* phylogeny. Furthermore, collectively we found associations among red and white colors and tubular flowers, with hummingbird pollination, while hypocrateriform shape was associated with butterfly pollination. For morphometric traits, we found an association between corolla length and nectar volume, and between corolla width and entrance size. The patterns of covariance among floral traits showed that floral integration is mainly associated with pollinators, although developmental associations cannot be not excluded, since there are allometric changes in some traits. Similar to Benitez-Vieyra *et al.* (2019), we found that there was no association among morphospace and covariance space, suggesting similar processes involved in morphological integration, such as pollinator-mediated selection. Our results also suggest that there is an interaction between pollinated mediated selection and developmental patterns that underlies floral trait associations in pollination syndrome definitions in *Achimenes*.

Literature

Abrahamczyk, S., Kessler, M., Hanley, D., Karger, D.N., Müller, M.P.J., Knauer, A.C., *et al.* 2017. Pollinator adaptation and the evolution of floral nectar sugar composition. *J. Evol. Biol.* **30**: 112–127.

Abrahamczyk, S., Souto-Vilarós, D. & Renner, S.S. 2014. Escape from extreme specialization: passion flowers, bats and the sword-billed hummingbird. *Proc. R. Soc. B* **281**: 20140888.

Albert, N.W., Davies, K.M. & Schwinn, K.E. 2014. Gene regulation networks generate diverse pigmentation patterns in plants. *Plant Signal. Behav.* **9**: 1–3.

Alcántara, S., de Oliveira, F.B. & Lohmann, L.G. 2013. Phenotypic integration in flowers of Neotropical lianas: diversification of form with stasis of underlying patterns. *J. Evol. Biol.* **26**: 2283–2296.

Anderson, I. & Busch, J.W. 2006. Relaxed pollinator-mediated selection in self-compatible taxa of *Leavenworthia* (Brassicaceae). *Am. J. Bot.* **93**: 860–867.

Andersson, S., Nilsson, L.A., Groth, I. & Bergström, G. 2002. Floral scents in butterfly-pollinated species: possible convergence in chemical composition. *Bot. J. Linn. Soc.* **140**: 129–153.

Armbruster, W.S. 2014. Floral specialization and angiosperm diversity: phenotypic divergence, fitness trade-offs and realized pollination accuracy.*AoB Plants* **6**

Armbruster, W.S., Di Stilio, V.S., Tuxill, J.D., Flores, T.C., Velazquez-Runk, J.L., Tuxtill, J.D., *et al.* 1999. Covariance and decoupling of floral and vegetative traits in nine Neotropical plants: a re-evaluation of Berg's correlation-pleiades concept. *Am. J. Bot.* **86**: 39–55.

Armbruster, W.S., Hansen T.F., Pélabon, C., Pérez-Barrales, R. & Maad, J. 2009. The adaptive accuracy of flowers: measurement and microevolutionary patterns. *Annals of Botany* **103**: 1545–2009.

Armbruster, W.S., Pélabon, C., Bolstad, G.H. & Hansen, T.F. 2014. Integrated phenotypes: Understanding trait covariation in plants and animals. *Philos. Trans. R. Soc. B Biol. Sci.* **369**: 20130245.

Ashman, T.L. & Majetic, C.J. 2006. Genetic constraints on floral evolution: A review and evaluation of patterns. *Heredity* **96**: 343–352.

Ashworth, L., Aguilar, R., Martén-Rodríguez, S., Lopezaraiza-Mikel, M., Avila-Sakar, G., Rosas-Guerrero, V., *et al.* 2015. Pollination syndromes: a global pattern of convergent evolution driven by the most effective pollinator. In: *Evolutionary Biology: Biodiversification from Genotype to Phenotype* (P. Pontarotti, ed.), pp. 203–224. Springer International Publishing, Switzerland.

Alexandersson, R. & Johnson, S.D. 2002. Pollinator-mediated selection on flower-tube length in a hawkmoth-pollinated *Gladiolus* (Iridaceae). *Proc. R. Soc. Lond. B.* **269**: 631–636.

Baranzelli, M.C., Benítez-Vieyra, S., Glinos, E., Trenchi, A., Córdoba, S., Camina, J., Ashworth, L., Sérsic, A.N., Cocucci, A.A. & Fornoni, J. 2020. Daily fluctuations in pollination effectiveness explain higher efficiency of native over exotic bees in *Lepechinia floribunda* (Lamiaceae). *Annals of Botany* **125**: 509–520.

Barrett, S.C.H. 2013. The evolution of plant reproductive systems: How often are transitions irreversible? *Proc. R. Soc. B Biol. Sci*. **280**: 20130913.

Benitez-Vieyra, S., Fornoni, J., Pérez-Alquicira, J, Boege, K. & Domínguez, C.A. 2014. The evolution of signal-reward correlations in bee- and hummingbird-pollinated species of *Salvia*. *Proc R. Soc. B* **281**: 20132934.

Benitez-Vieyra, S., Pérez-Alquicira, J., Sazatornil, F.D., Domínguez, C.A., Boege, K., Pérez-Ishiwara, R., *et al.* 2019. Evolutionary transition between bee pollination and hummingbird pollination in *Salvia*: comparing means, variances and covariances of corolla traits. *J. Evol. Biol.* **32**: 783–793.

Berg, R.L. 1959. A general evolutionary principle underlying the origin of developmental homeostasis. *Am. Nat.* **93**: 103–105.

Berg, R.L. 1960. The ecological significance of the correlation pleiades. *Evolution* **14**: 171–180.

Beutelspascher, C.R. & Martínez-Meléndez J. 2008. Una nueva especie de Achimenes Persoon, (Gesneriaceae) de Chiapas, México. *Lacandonia* **2**: 5–10.

Bradley, J.M., Davies, K. M., Deroles, S. C., Bloor, S. J., Lewis, D. H. 1998. The maize Lc regulatory gene up-regulates the flavonoid biosynthetic pathway of *Petunia*. *Plant J.* **13**: 381–392.

Bradshaw Jr., H.D. & Schemske, D.W. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* **426**: 176–178.

Bukovac, Z., Shrestha, M., Garcia, J.E., Burd, M., Dorin, A. & Dyer, A.G. 2017. Why background colour matters to bees and flowers. *J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol.* **203**: 369–380.

Butler, M.A. & King, A.A. 2004. Phylogenetic comparative analysis: a modeling approach for adaptive evolution. *Am. Nat.* **164**: 683–695.

Cazonelli, C.I. 2011. Carotenoids in nature: insights from plants and beyond. *Func. Plant Biol*. **38**: 833–847.

Cressler, C.E., Butler, M.A. & King, A.A. 2015. Detecting adaptive evolution in phylogenetic comparative analysis using the Ornstein-Uhlenbeck model. *Syst. Biol. **64***: 953–968.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. 2012. jModelTest2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772.

Dellinger, A.S., Artuso, S., Pamperl, S., Michelangeli, F.A., Penneys, D.S., Fernández-Fernández, D.M, Alvear, M., *et al*. 2019. Modularity increases rate of floral evolution an adaptive success for functionally specialized pollination systems. *Nat. Comm. Biol.* **2**: 1-11.

De Luca, P.A. & Vallejo-Marín, M. 2013. What's the buzz about? The ecology and evolutionary significance of buzz pollination. *Curr. O. Plant Biol.* 16: 429–435.

Dodd, M.E., Silvertown, J. & Chase, M.W. 1999. Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families. *Evolution* **53**: 732–744.

Doyle, J.J. & Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**: 11–15.

Dressler, R.L. 1968. Pollination by Euglossine bees. *Evolution* **22**: 202–210.

Durbin, M.L., Lundy, K.E., Morrell, P.L., Torres-Martinez, C.L. & Clegg, M.T. 2003. Genes that determine flower color: The role of regulatory changes in the evolution of phenotypic adaptations. *Mol. Phylogenet. Evol.* **29**: 507–518.

Esteve-Altava, B. 2017. In search of morphological modules: a systematic review. *Biol. Rev.* **92**: 1332 –1347.

Felice, R.N., Randau, M., & Goswami, A. 2019. A fly in a tube: macroevolutionary expectations for integrated phenotypes. *Evolution* **72**: 2580–2594.

Felsenstein, J. 1985. Phylogenies and the comparative method. *Am. Nat.* ***125***: 1–15.

Fenster, C.B., Armbruster, W.S., Wilson, P., Dudash, M.R. & Thomson, J.D. 2004. Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* **35**: 375–403.

Fenster, C.B. & Martén-Rodríguez, S. 2007. Reproductive assurance and the evolution of pollination specialization. *Int. J. Plant Sci.* **168**: 215–228.

Fenster, C.B., Reynolds, R.J., Williams, C. W., Makowsky, R. & Dudash, M.R. 2015. Quantifying hummingbird preference: for floral trait combinations: the role of selection on trait interactions in the evolution of pollination syndromes. *Evolution* **69**: 1113–1127.

Fornoni, J., Boege, K., Domínguez, C.A. & Ordano, M. 2008. How little is too little? The adaptive value of floral integration. *Commun. Integr. Biol.* **1**: 56–58.

Fornoni, J., Ordano, M., Pérez-Ishiwara, R., Boege, K. & Domínguez, C.A. A comparison of floral integration between selfing and outcrossing species: a meta-analysis. 2015. *Annals of Botany* **117**: 299–306.

Garland Jr., T. & Ives, A.R. 2000. Using the past to predict the present: confidence intervals for regression equations in phylogenetic comparative methods. *Am. Nat.* **155**: 346–364.

Goolsby, E.W. 2015. Phylogenetic comparative methods for evaluating the evolutionary history of function-valued traits. *Sist. Biol.* **64**: 568–578.

Gómez, J.M., Perfectti, F. & Klingenberg, C.P. 2014. The role of pollinator diversity in the evolution of corolla-shape integration in a pollination-generalist plant clade. *Philos. Trans. R. Soc. B Biol. Sci.* **369**.

Gómez, J.M., Torices, R., Lorite, J., Klingenberg, C.P. & Perfectti, F. 2016. The role of pollinators in the evolution of corolla shape variation, disparity and integration in a highly diversified plant family with a conserved floral bauplan. *Ann. Bot.* **117**: 889–904.

Goswami, A., Smaers, J.B., Soligo, C. & Polly, D. 2014. The macroevolutionary consequences of phenotypic integration: from development to deep time. *Phil. Trans. R. Soc. B* **369**: 20130254.

Grafen, A. 1989. The phylogenetic regression. *Philos. Trans. R. Soc. B Biol. Sci.* **326**: 119–157.

Harmon, L.J., Weir, J.T., Brock, C.D., Glor, R.E. & Challenger, W. 2008. GEIGER: Investigating evolutionary radiations. *Bioinformatics* **24**: 129–131.

Joly, S., Alexandre, H., Clavel, J., Clark, J.L., Lambert, F., Alexandre, H., *et al.* 2018. Greater pollination generalization is not associated with reduced constraints on corolla shape in Antillean plants. *Evolution.* **72**: 244–260.

Joy, J.B., Liang, R.H., McCloskey, R.M., Nguyen, T. & Poon, A.F.Y. 2016. Ancestral Reconstruction. *PLOS Comput. Biol.* **12**: e1004763.

Katoh, K., Rozewicki, J. & Yamada, K.D. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* bbx108.

Kay, K.M., Reeves, P.A., Olmestead, R.G. & Schemske, D.W. 2005. Rapid speciation and the evolution of hummingbird pollination in Neotropical Costus subgenus Costus (Costaceae): evidence from nrDNA ITS and ETS sequences. *Am. J. Bot.* **92**: 1899–1910.

Kay, K.M. & Sargent, R.D. 2009. The role of animal pollination in plant speciation: integrating Ecology, Geography, and Genetics. *Annu. Rev. Ecol. Evol. Syst.* **40**: 637–656.

King, A.A. & Butler, M.A. 2019. ouch: Ornstein-Uhlenbeck models for phylogenetic comparative hypotheses (R package).

Koski, M.H. & Ashman, T. 2014. Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Func. Ecol.* **28**: 868–877.

Kumar S, Stecher G, Li M, Knyaz C, and Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol.Evol.* 35:1547-1549.

Lagomarsino, L.P., Forrestel, E.J., Muchhala, N., Davies, C.C. 2017. Repeated evolution of vertebrate pollination syndromes in a recently diverged Andean plant clade. *Evolution* **71-8**: 1970–1985.

Landis, J.B., Bell, C.D., Hernandez, M., Zenil-Ferguson, R., McCarthy, E.W., Soltis, D.E., *et al.* 2018. Evolution of floral traits and impact of reproductive mode on diversification in the phlox family (Polemoniaceae). *Mol. Phylogenet. Evol.* **127**: 878–890.

Lara, C. & Ornelas, J.F. 2008. Pollination ecology of *Penstemon roseus* (Plantaginaceae), an endemic perennial shifted toward hummingbird pollination? *Pl. Syst. Evol.* **271**: 223–237.

Lázaro, A. & Santamaría, L. 2015. Flower visitor selection on floral integration in three contrasting populations of *Lonicera implexa*. *Am. J.Bot.* **103**: 325–336.

Losos, J.B. 2011. Convergence, adaptation, and constraint. *Evolution* **65**: 1827–1840.

Martén-Rodríguez, S., Almarales-Castro, A. & Fenster, C.B. 2009. Evaluation of pollination syndromes in Antillean Gesneriaceae: evidence for bat, hummingbird and generalized flowers. *J. Ecol.* **97**: 348–359.

Martén-Rodríguez, S., Fenster, C.B., Agnarsson, I., Skog, L.E. & Zimmer, E.A. 2010. Evolutionary breakdown of pollination specialization in a Caribbean plant radiation. *New Phytol.* **188**: 403–417.

Martínez del Río, C. 1990. Sugar preferences in hummingbirds: the influence of subtle chemical differences on food choice. *Condor* **92**: 1022–1030.

Martin, C. & Gerats, T. 1993. Control of pigment biosynthesis genes during petal development. *Plant Cell* **5**: 1253–1264.

Melo, D., García, G., Hubbe, A., Assis, A.P. & Marroig, G. 2015. EvolQG- an R package for evolutionary quantitative genetics [version 3; referees: 2 approved, 1 approved with reservations. *F1000Research* **4**: 1–24.

Miller, M.A., Pfeiffer, W., and Schwartz, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp 1–8.

Montgomerie, R.D. 1984. Nectar extraction by hummingbirds: response to different floral characters. *Oecologia* **63**: 229–236.

Muchhala, N. 2003. Exploring the boundary between pollination syndromes: bats and hummingbirds as pollinators of *Burmeistera cyclostigmata* and *B. tenuiflora* (Campanulaceae). *Oecologia* **134**: 373–380.

Muchhala, N., Caiza, A., Vizuete, J.C. & Thomson, J.D. 2009. A generalized pollination system in the tropics: bats, birds and *Aphelandra acanthus*. *Ann. Bot.* **103**:1481–1487.

Müller, J., Müller, K., Neinhuis, C., & Quandt, D. 2010. PhyDE, Phylogenetic Data Editor, version v0.9971.

Mundry, R. 2014. Statistical issues and assumptions of phylogenetic generalized least squares. In. *Modern phylogenetic comparative methods and their application* (Zsolt-Garamszegi L, ed.). Springer-Verlag, Berlin Heidelberg. Pp 131–153.

Newman, E., Manning, J. & Anderson, B. 2015. Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities.

Evolution **69**: 2262–2275.

O’Meara, B.C., Smith, S.D., Armbruster, W.S., Harder, L.D., Hardy, C.R., Hileman, L.C., Hufford, L., Litt, A., Magallón, S., Smith, S.A., Stevens, P.F., Fenster, C.B. & Diggle, P.K. 2016. Non-equilibrium dynamics and floral trait interactions shape extant angiosperm diversity. *Proc. R. Soc. B* **283**: 20152304.

Ordano, M., Fornoni, J., Boege, K. & Domínguez, C.A. 2008. The adaptive value of phenotypic floral integration. *New Phytol.* **179**: 1183–1192.

Ornelas, J.F. & Ordano, M. 2007. Nectar removal effects on seed production in *Moussonia deppeana* (Gesneriaceae), a hummingbird-pollinated shrub. *Ecoscience* **14**: 117–123.

Ornelas, J.F., Ordano, M., De-nova, J.A., Quintero, M.E. & Garland, T. 2007. Phylogenetic analysis of interspecific variation in nectar of hummingbird-visited plants. *J. Evol. Biol.* **20**: 1904–1917.

Pagel, M. 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. *Proc. R. Soc. London, B* **255**: 37–45.

Pagel, M. & Meade, A. 2006. Bayesian analysis of correlated evolution of discrete characters by Reversible-Jump Markov Chain Monte Carlo. *Am. Nat.* **167**: 808–825.

Paradis, E., & Schliep, K. 2019. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* **35**: 526–528.

Parachnowitsch, A.L., & Caruso, C. M. 2008. Predispersal seed herbivores, not pollinators, exert selection on floral traits via female fitness. *Ecology* **89**: 802–1810.

Pavlicev, M., Cheverud, J.M. & Wagner, G.P. 2009. Measuring morphological integration using eigenvalue variance. *Evol. Biol.* **36**: 157–170.

Pérez-Barrales, R., Simón-Porcar, V.I., Santos-Gally, R. & Arroyo, J. 2014. Phenotypic integration in style dimorphic daffodils (*Narcissus*, Amaryllidaceae) with different pollinators. *Philos. Trans. R. Soc. B Biol. Sci*. **369**: 20130258.

Pérez, F., Arroyo, M.T.K. & Medel, R. 2007. Phylogenetic analysis of floral integration in *Schizanthus* (Solanaceae): does pollination truly integrate corola trait?. *J. Evol. Biol.* **20**: 1730–1738.

Pinheiro, J., Bates, D., DebRoy, S. & Sarkar, D. 2019. nlme: Linear and Nonlinear Mixed Effects Models. RCore Team (2019).

Plummer, M., Best, N., Cowles K. & Vines K. 2006. CODA: Convergence Diagnosis and Output Analysis for MCMC, *R News* **6**: 7-11.

R-Core-Team. 2019. R: a language and environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramírez-Aguirre E. 2011. Integración floral de *Moussonia deppeana* (Schlechtend & Cham) Hanst y su relación con los rasgos de néctar y éxito reproductivo. Bachelor Thesis, Biology Faculty, Universidad Veracruzana.

Ramírez-Aguirre, E., Martén-Rodríguez, S. & Ornelas, J.F. 2016. Floral variation, nectar production, and reproductive success of two *Drymonia* (Gesneriaceae) species with mixed pollination syndromes. *Int. J. Plant Sci.* **177**: 469–480.

Ramírez-Aguirre, E., Martén-Rodríguez, S., Quesada-Avila, G., Quesada, M., Martínez-Díaz, Y., Oyama, K. & Espinosa-García, F.J. 2019. Reproductive isolation among three sympatric *Achimenes* species: pre- and post-pollination components. *Am. J. Bot.* **106**: 1021–1031.

Ramírez-Roa, A. & Skog, L.E. 2002. Novae Gesneriaceae Neotropicarum X: A new species of *Achimenes* from Mexico. *Novon* **12**: 382–384.

Ramírez-Roa, M.A. de la P. 1987. Revisión de *Achimenes* (Gesneriaceae). Bachelor Thesis. Faculty of Sciences, Universidad Nacional Autónoma de México.

Rausher, M.D. 2008. Evolutionary transitions in flower color. *Int. J. Plant Sci.* **169**: 7–21.

Revell, L.J., Harmon, L.J. & Collar, D.C. 2008. Phylogenetic signal, evolutionary process, and rate. *Syst. Biol.* **57**: 591–601.

Revell, L.J. 2012. phytools: An R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* **3**: 217–223.

Reich, D., Berger, A., von Balthazar, M., Chartier, M., Sherafati, M., Shönenberger, J., Manafzadeh, S. & Staedler, Y. M. 2020. Modularity and the evolution of floral shape: the role of function, development and spandrels in *Erica*. *New Phytol*. **226**: 267–280.

Reynolds, R.J., Dudash, M.R. & Fenster, C.B. 2009. Multiyear study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated Silene virginica. *Evolution* **64**: 358–369.

Roalson, E.H., Skog, L.E. & Zimmer, E.A. 2003. Phylogenetic relationships and the diversification of floral form in *Achimenes* (Gesneriaceae). *Syst. Bot.* **28**: 593–608.

Roberts, W.R. & Roalson, E.H. 2018. Phylogenomic analyses reveal extensive gene flow within the magic flowers (*Achimenes*). *Am. J. Bot.* **105**: 726–740.

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J.M., *et al.* 2014. A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett.* **17**: 388–400.

Rosas-Guerrero, V., Quesada, M., Armbruster, W.S., Pérez-Barrales, R. & Smith, S.D.W. 2011. Influence of pollination specialization and breeding system on floral integration and phenotypic variation in ipomoea. *Evolution* **65**: 350–364.

SanMartin-Gajardo, I. & Sazima, M. 2005. Chiropterophily in Sinningieae (Gesneriaceae): *Sinningia brasiliensi*s and *Paliavana prasinata* are bat-pollinated, but *P. sericiflora* is not. Not yet? *Ann. Bot.* **95**: 1097–1103.

Schiestl, F.P. & Johnson, S.D. 2013. Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* **28**: 307–315.

Serrano-Serrano, M., Perret, M., Guignard, M., Chautems, A., Silvestro, D. & Salamin, N. 2015. Decoupled evolution of floral traits and climatic preferences in a clade of Neotropical Gesneriaceae. *BMC Evol. Biol.* **15**: 247.

Sheehan, H., Moser, M., Klahre, U., Esfeld, K., Dell'olivo, A., Mandel, T., *et al.* 2016. MYB-FL controls gain and loss of floral UV absorbance, a key trait affecting pollinator preference and reproductive isolation. *Nat. Genet.* **48**: 159–166.

Shrestha, M., Dyer, A.G., Boyd-Gerny, S., Wong, B.B.M. & Burd, M. 2013. Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New Phytol.* **198**: 301–310.

Smith, S.D. 2016. Pleiotropy and the evolution of floral integration. *New Phytol.* **209**:80–85.

Smith, S.D.W., Ané, C. & Baum, D.A. 2008. The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution* **62**: 793–806.

Smith, S.D.W & Kriebel, R. 2018. Convergent evolution of floral shape tied to pollinator shifts in Iochrominae (Solanaceae). *Evolution* **72**: 688–697.

Specht, C.D. & Howarth, D.G. 2015. Adaptation in flower form: a comparative evodevo approach. *New Phytol.* **206**: 74–90.

Stebbins, G.L. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. *Annu. Rev. Ecol. Syst.* **1**: 307–326.

Strauss, S.Y. & Whittall, J.B. 2006. Non-pollinator agents of selection. In: *Ecology and Evolution of Flowers* (L. D. Harder & S. C. H. Barrett, eds.), pp. 120–135. Oxford University Press, New York.

Strelin, M.M., Benitez-Vieyra, S., Ackermann, M. & Cocucci, A.A. 2016a. Flower reshaping in the transition to hummingbird pollination in Loasaceae subfam. Loasoideae despite absence of corolla tubes or spurs. *Evol. Ecol.* **30**: 401–417.

Temeles, E.J. 1996. A new dimension to to hummingbird-flower relationships. *Oecologia* **105**: 517–523.

Toon, A., Cook, L.G. & Crisp, M.D. 2014. Evolutionary consequences of shifts to bird-pollination in the Australian pea-flowered legumes (Mirbelieae and Bossiaeeae). *BMC Evol. Biol.* **14**: 1–11

Torices, R., & Muñoz-Pajares, A. J. 2015. PHENIX: An R package to estimate a size-controlled phenotypic integration index. *Applications in plant sciences* **3**: 1400104.

Tripp, E.A. & Manos, P.S. 2008. Is floral specialization an evolutionary dead-end? Pollination system transitions in *Ruellia* (Acanthaceae). *Evolution* **62**: 1712–1737.

Valente, L.M., Manning, J.C., Goldblatt, P. & Vargas, P. 2012. Did pollination shifts drive diversification in Southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *Am. Nat.* **180**: 83–98.

Weber, A., Clark, J.L. & Möller, M. 2013. A new formal classification of Gesneriaceae. *Selbyana* **31**: 68–94.

Wessinger, C.A., Hileman, L.C. & Rausher, M.D. 2014. Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Phil. Trans. R. Soc.* **369**: 20130349.

Wessinger, C.A. & Hileman, L.C. 2016. Accessibility, constrain, and repetition in adaptive floral evolution. *Dev. Biol.* **419**: 175–183.

Whittal, J.B. & Hodges, S.A. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**: 706–709.

Wiehler, H. 1976. A report on the classification of *Achimenes*, *Eucodonia*, *Gloxinia*, *Goyazia*, and *Anetanthus* (Gesneriaceae). *Selbyana* **1**: 374–401.

Wiehler, H. 1992. New species of Gesneriaceae from the Neotropics. *Phytologia* **73**: 220–241.

Wilson, P., Wolfe, A.D., Armbruster, S.W. & J.D. Thomson. 2007. Constrained lability in floral evolution: counting convergent origins of hummingbird pollination in *Penstemon* and *Keckiella*. *New Phytologist* **176**: 883–890.

Wilson, P., Castellanos, M.C., Wolfe, A.D. & J.D. Thomson. 2006. Shifts between bee and bird-pollination among penstemons. In *Specialization and generalization in pollination systems* (Waser, N.M. & Ollerton, J., eds.). University of Chicago Press.

Whittall, J.B. & Hodges, S.A. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**: 706–710.

Wózniak, N.J. & Sicard, A. 2018. Evolvability of flower geometry: convergence in pollinator-driven morphological evolution of flowers. *Sem. Cell. & Dev. Biol.* **79**: 3–15.

Yong, R.L. & Badyaev, A.V. 2006. Evolutionary persistence of phenotypic integration: influence of developmental and functional relationships on complex trait evolution. *Evolution* **60**: 1291–1299.

Yuan, Y-W., Sagawa, J.M., Di Stilio, V.S. & Bradshaw, H.D.Jr. 2013. Bulk segregant analysis of an induced floral mutant identifies a *MIXTA*-like R2R3 MYB controlling nectar guide formation in *Mimulus lewisii*. *Genetics* **194**: 709–719.

Zhong, J. & Preston, J.C. 2015. Bridging the gaps: evolution and development of perianth fusion. *New Phytol.* **208**: 303–305.

Tables and figures

Table 1. Pollinator syndromes and observed pollination systems of *Achimenes* and other Gesneriaceae species in Mexico, during 2013-2018. The legend "N.o." indicates not-observed.

Species	**Pollination syndrome**	**Observed main pollinators**	**Mean visits /hr**	**Total hours**	**Other floral visitors**	**Nº of plants/ patch**	**Nº of populations**
A. admirabilis	Butterfly	Small bee	0.21	8	N.o.	1-30	1
A. antirrhina	Hummingbird	Hummingbird	0.6	5	N.o.	5-50	1
A. candida	Small bee	Small bee	0.8	12	Butterfly	3-20	2
A. cettoana	Butterfly	Butterfly	0.22	8	Bees	3-50	2
A. dulcis	Large bee	Large bee	1.6	13	N.o.	5-20	1
A. erecta	Butterfly	Butterfly	0.1	15	N.o.	5-200	2
A. fimbriata	Small bee	Small bee	2.16	8	N.o.	1- 100	2
A. flava	Large bee	Med-large bees	0.16	6	N.o.	3-50	1
A. grandiflora	Butterfly	Butterfly	0.56	8	Small bees	7-20	2

A. glabrata	Large bee	Not observed		3	N.o.	15	1
A. heterophylla	Hummingbird	Hummingbird	1.06	18	Small bees*	10-50	3
A. hintoniana	Large bee	Large bee	0.9	9	Butterfly	3-40	2
A. longiflora	Butterfly	Butterfly	0.13	15	Large bee*	3-20	3
A. mexicana	Large bee	Large bee	1.6	10	N.o.	5-50	1
A. misera	Small bee	Small bee	0.7	16	N.o.	5-30	1
A. obscura	Small bee	Small bee	1.6	3	N.o.	8-30	1
A. occidentalis	Small bee	Butterfly	0.6	12	N.o.	20-50	2
A. patens	Butterfly	Butterfly	0.5	8	N.o.	10-30	1
A. pedunculata	Hummingbird	Hummingbird	0.5	25	Small bee*	10-60	2
A. skinneri	Hummingbird	Hummingbird	0.9	13	N.o.	50	1
A. woodii	Small bee	Small bee	1.4	12	Butterfly	10-100	1

A. aff. ixtapaensis	Small bee	Not observed			N.o.		
A. sp.1	Hummingbird	Not observed		2	N.o.	50	1
A. sp. 2	Small bee	Small bee			N.o.	50	1
Moussonia deppeana	Hummingbird	Ordano & Ornelas 2007	N.o.	N.o.	Large bee, pers. obs.*	N.o.	1
Eucodonia verticillata	Large bee	Large bee	N.o.	N.o.		10	1
Smithiantha aff. cinnabarina	Hummingbird	Not observed	N.o.	N.o.	N.o.	N.o.	1

*Asterisks indicate floral visitors that were observed robbing nectar or pollen from flowers

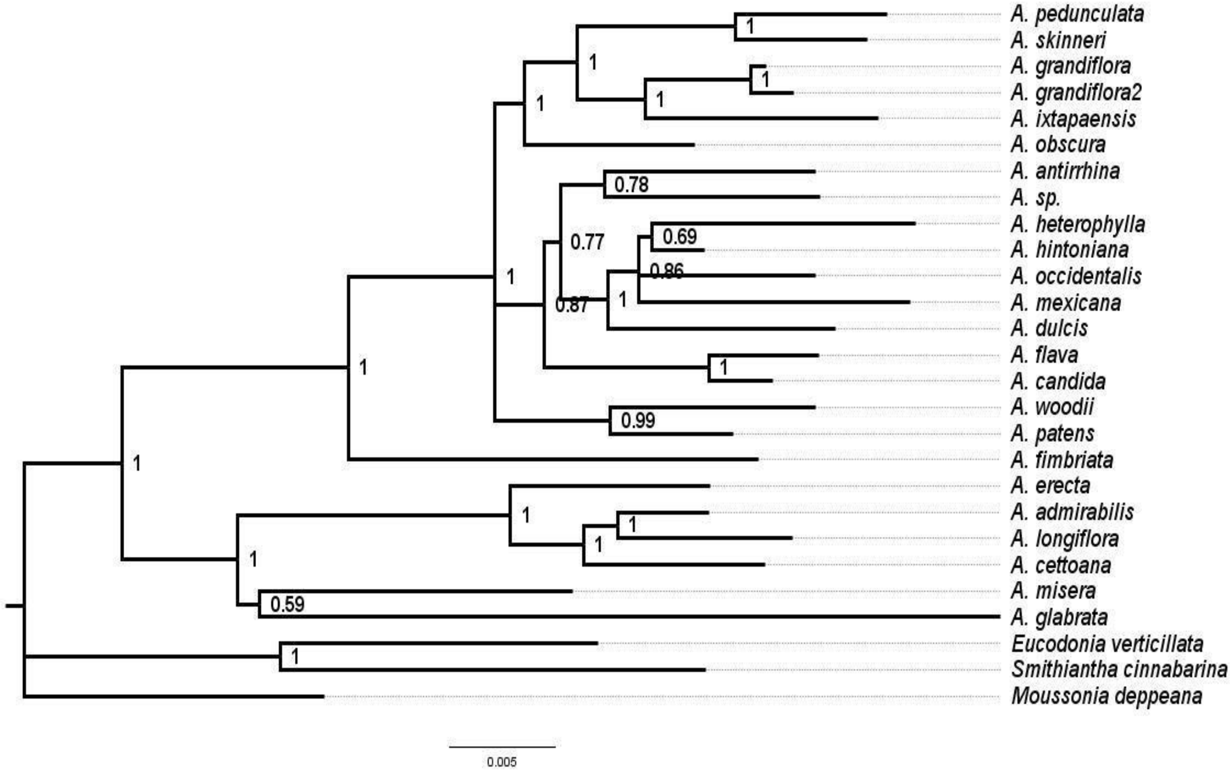

Figure 1. Consensus tree from phylogenetic Bayesian inference with five nuclear markers (GCYC, ETS, ITS complete sequence, trnL-trnF, and rpl32-trnL) of *Achimenes* species and three outroups of Gesneriaceae. Numbers represent posterior probabilities.

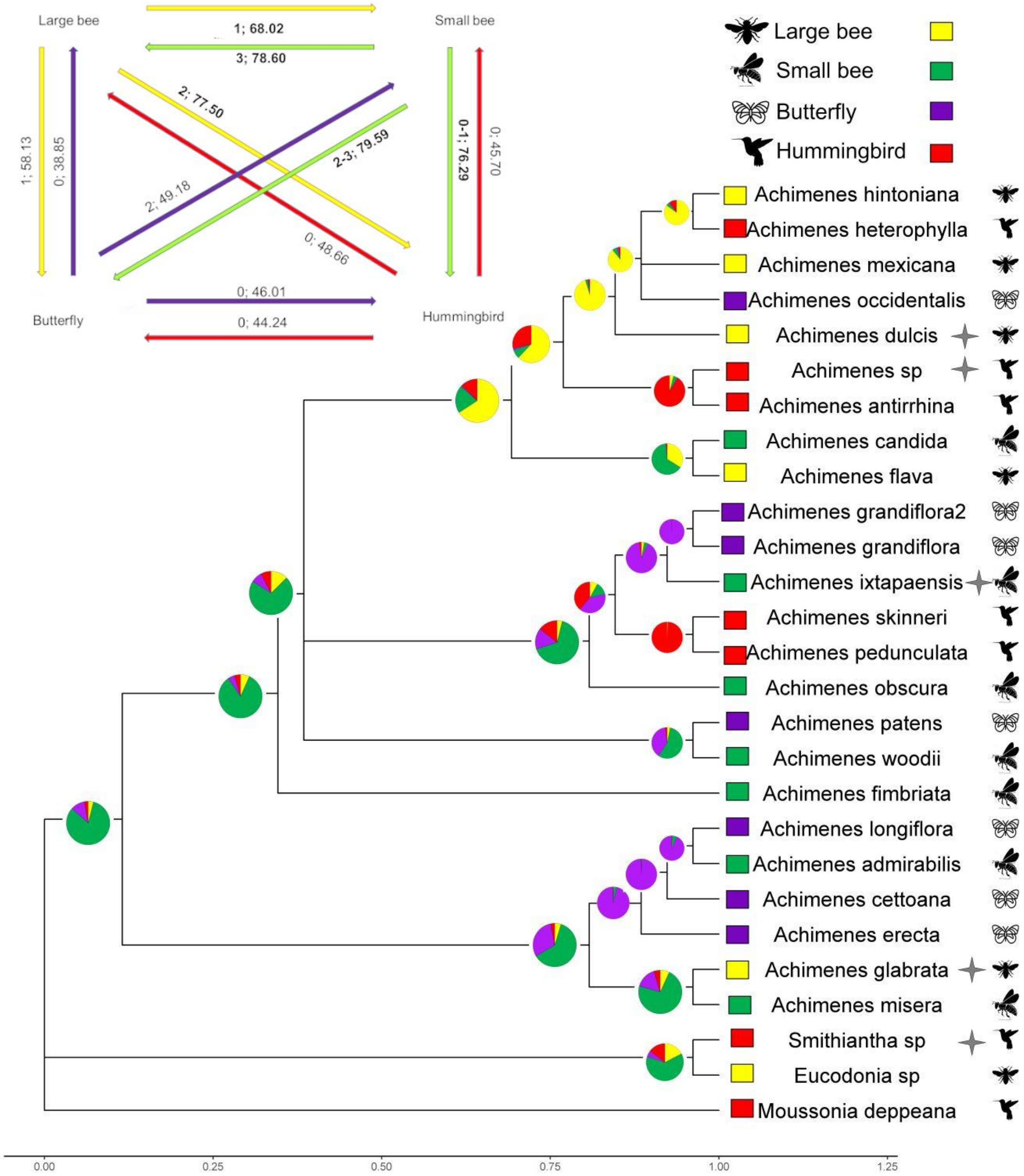

Figure 2. Pollination systems ancestral reconstruction in *Achimenes* species and three outgroups. The star next to animal figures represents inferred pollination systems based on pollination syndromes. Pies represent mean probabilities for each pollination system and colors represent different pollinators. Top left figure shows mean transition rates between pollination systems based posterior values from MULTISTATE model in BayesTraits. The first number represents the observed frequency of transitions and the second represents the mean transition rate based on the distribution of posterior probability.

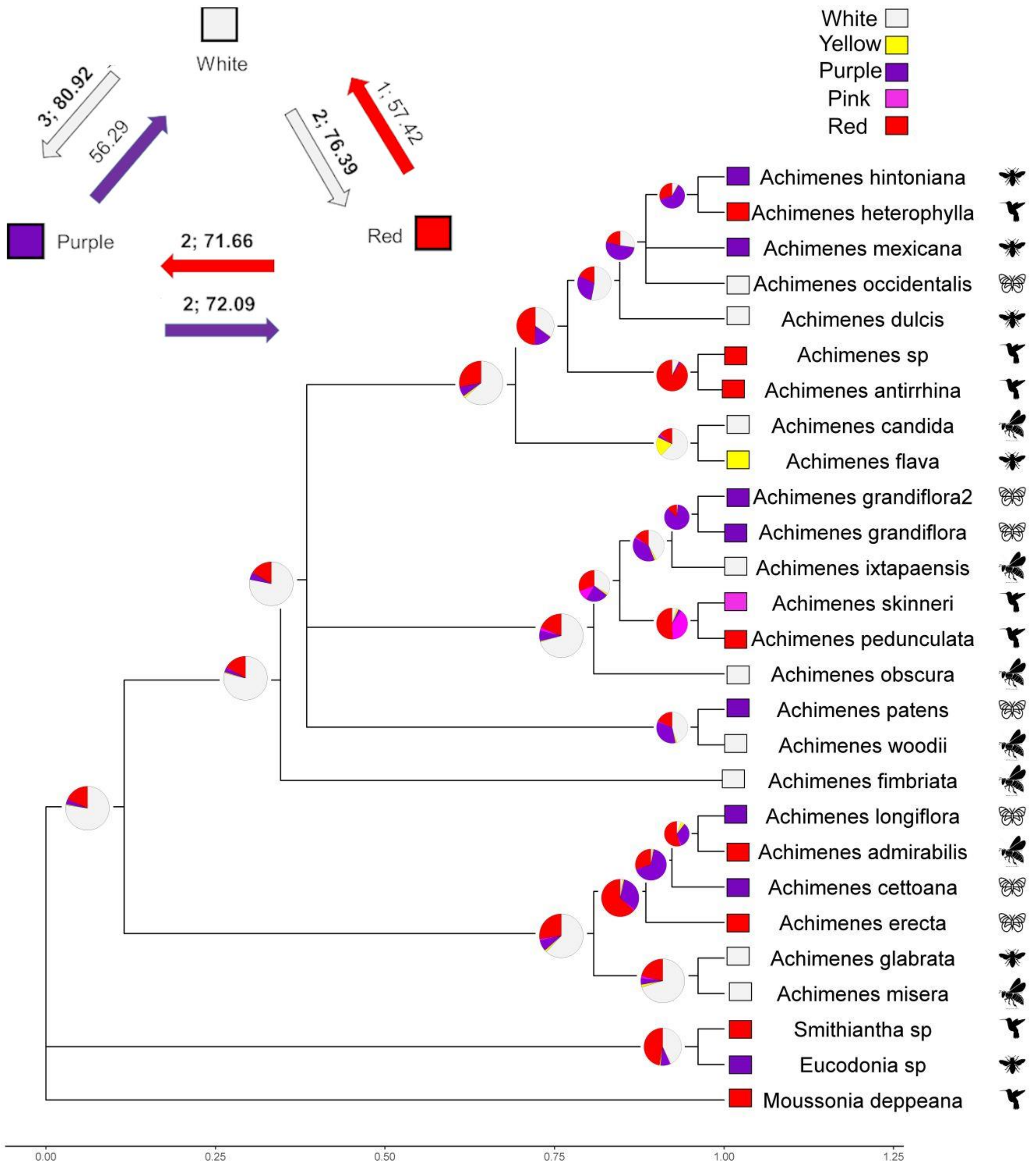

Figure 3. Corolla colors ancestral reconstruction and pollinators in *Achimenes* species and three outgroups. The animal figures represent, either pollination systems based on observed pollinators, and the star next to animal figures represents inferred pollination systems based on pollination syndromes. Pies represent mean posterior probabilities for corolla color states. Top left figure shows mean transition rates between pollination systems based posterior values from MULTISTATE model in BayesTraits. The first number represents the observed frequency of transitions and the second represents the mean transition rate based on the distribution of posterior probability.

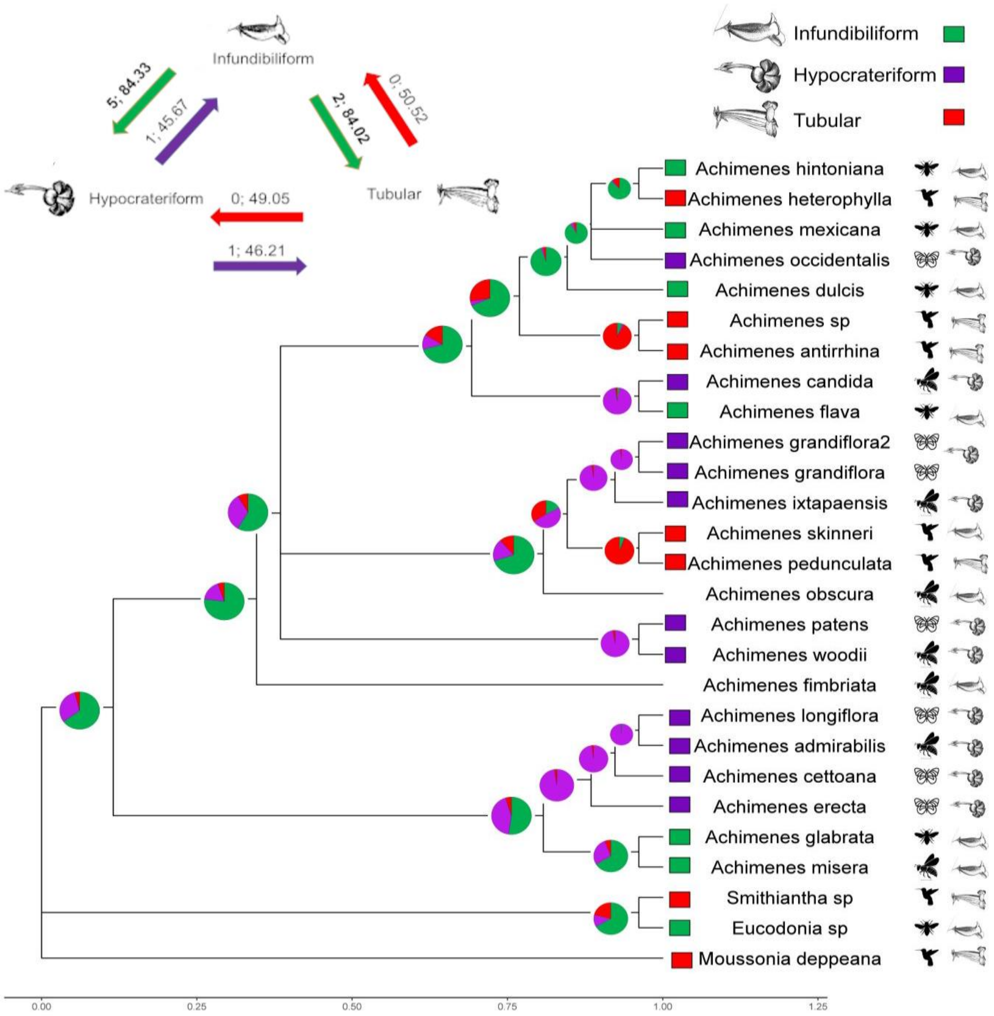

Figure 4. Corolla shape ancestral reconstruction and pollinator systems of *Achimenes* species and three outgroups. The animal figures represent, either pollination systems based on observed pollinators, and the star next to animal figures represents inferred pollination systems based on pollination syndromes. Pies represent mean posterior probabilities for corolla color states (green = infundibiliform; purple = hypocrateriform; red = tubular). Top left figure shows mean transition rates between pollination systems based posterior values from MULTISTATE model in BayesTraits. The first number represents the observed frequency of transitions and the second represents the mean transition rate based on the distribution of posterior probability.

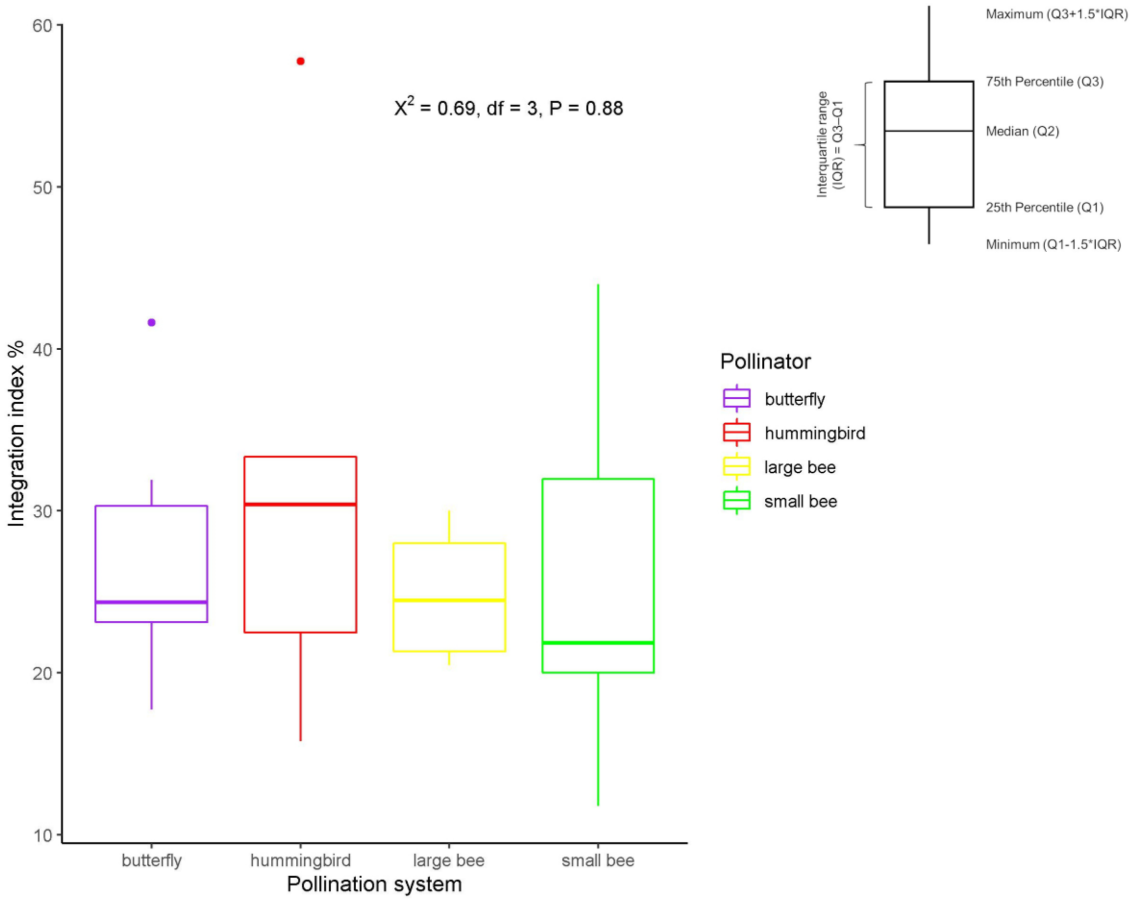

Figure 5. Floral integration indexes of 21 *Achimenes* species depicted by pollination system. Chi-squared and probability are the results of a Kruskall-Wallis test of association between Floral Integration Index (%) and pollination system.

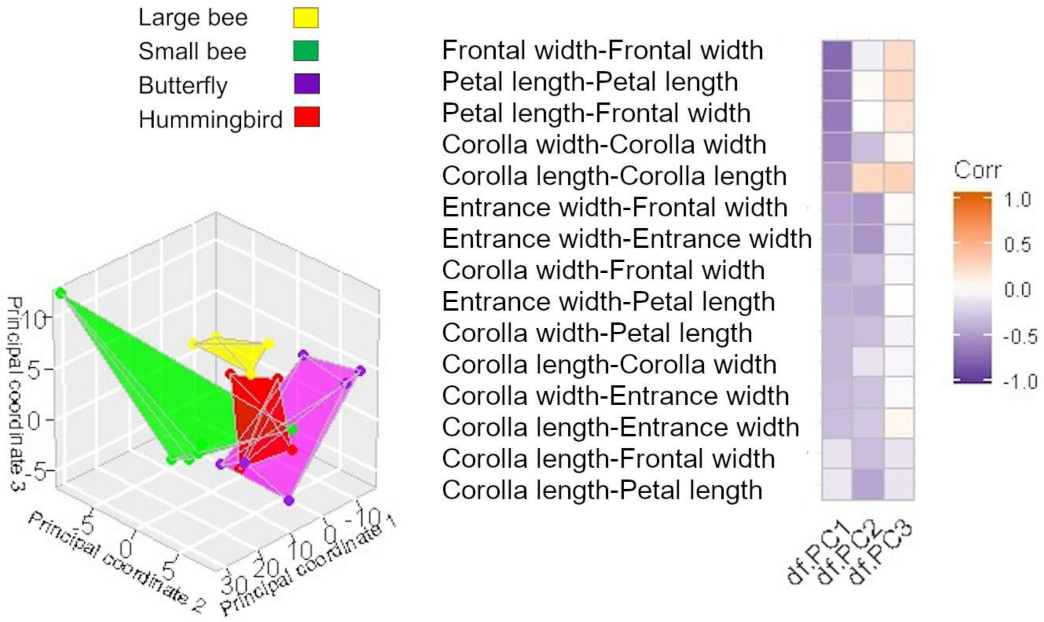

Figure 6. (a) Covariance space of *Achimenes* species. (b) Correlations between principal coordinates and variances.

Supporting Information

Supporting Information 1. CTAB 2X protocol for DNA extraction.

Depending on leaf size, approximately 30-50 µg of leaf tissue was grinded with liquid Nitrogen in sterilized mortars, and leaf dust was deposited in 1000µl tubes, previously identified with species name (Eppendorf, Germany). Then, 400-700 µl of CTAB 2X buffer was added to the tube with 10sec vortex and was incubated at 55°C for 24 hrs with occasional vortexing. After incubation time, 2 µl of proteinase K (Promega, Madison Wisconsin, US) was added and the mix was incubated at 56°C for 18 hrs. After incubation, tubes were centrifuged at 120000 revolutions per minute (rpm) for 5 minutes. Supernatant was transferred to new previously identified Eppendorf tubes at an approximate volume of 500 µl. Then, a 24:1 ratio of chloroform:isoamyl alcohol was added to the tubes, followed by vortexing for 20 sec. After vortexing, tubes were centrifuged at 130000 rpm for 5 min. Supernatant was transferred to a previously marked clean Eppendorf tube (transferred liquid volume was recorded) and 1 µl of RNase (Promega, Madison Wisconsin, US) was added for each 100 µl of supernatant. Tubes were incubated at 37°C for one hour. After incubation, an equal volume of isopropanol and 10% of total volume (alcohol and sample) of 3.5M Sodium Acetate was added to the tubes. Then, tubes were placed at -20°C during night. After freezing, tubes were centrifuged at 130000 rpm for 15 min. Pellet was washed with 80% cold ethanol 2-3 times and most alcohol was pipetted out. Residual alcohol drops in tubes were dried with a Concentrator ® Plus (Eppendorf, Germany) for two rounds of 3 minutes at 45°C, with the AL (alcohol) mode. Tubes were checked visually for residual alcohol. Finally, DNA was resuspended in 30-50 µl of ultrapure distilled water.

Amplification protocol

Polymerase chain reaction (PCR) mix was done for two 25 µl reactions per one individual per species, with 12.5 µl of Multiplex mix, 2.5 µl of each primer for individual markers, 5.5 µl of ultrapure and distilled water, and 2 µl of DNA sample. For trnL-trnF region, 2.5µl Q Solution ® were added, with 12.5 µl of Multiplex mix, 3µl of ultrapure and distilled water, 2.5µl of each primer, and 2µl of DNA sample to a final volume of 25 µl. Reactions were performed in either a Mastercycler nexus Gradient ® (Eppendorf, Germany) or a Veriti ® Thermal cycler (Applied Biosystems (™), Thermo Fisher Scientific Co., Waltham, MA, US). Individual marker PCR programs with respective primers and primer concentration are described in Table S1. After PCR, amplification was checked in 1.2% agarose gels stained with Diamond (™) SYBR ® nucleic acid dye (Promega, Madison Wisconsin, US).

Literature cited

Baldwin, B.G. & Markos, S. 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of ETS and ITS trees of *Calycadenia* (Compositae). *Mol. Phylogenet. Evol.* **10**: 449–463.

Beardsley, P.M. & Olmstead, R.G. 2002. Redefining Phrymaceae: the placement of *Mimulus* , tribe Mimuleae, and *Phryma*. *Am. J. Bot.* **89**: 1093–1102.

Dong, W., Liu, J., Yu, J., Wang, L. & Zhou, S. 2012. Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. *PLoS One* **7**: 1–9.

Doyle, J.J. & Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11–15.

Hershkovitz, M.A. & Zimmer, E.A. 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acids Res.* **24**: 2857–2867.

Möller, M., Clokie, M., Cubas, P. & Cronk, Q.C.B. 1999. Integrating molecular phylogenies and developmental genetics: a Gesneriaceae case study. In: *Molecular systematics and plant evolution* (P. M. Hollinsworth, R. M. Bateman, & R. J. Gornall, eds), pp. 375–402. Taylor & Francis, London, New York.

Shaw, J., Lickey, E.B., Schilling, E.E. & Small, R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in Angiosperms: the tortoise and the hare III. *Am. J. Bot.* **94**: 275–288.

Smith, J.F., Hileman, L.C., Powell, M.P. & Baum, D.A. 2004. Evolution of GCYC, a Gesneriaceae homolog of CYCLOIDEA, within Gesnerioideae (Gesneriaceae). *Mol. Phylogenet. Evol.* **31**: 765–779.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* **17**: 1105–1109.

White, T.J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White, eds), pp. 315–322. Academic Press.

Tables and figures

Table S1. Polymerase chain reaction (PCR) amplification conditions of five molecular markers for *Achimenes* species.

Region	**Primer name and sequence**	**PCR Program**	**Primer concentration**
ITS4-ITS5H	ITS4 (White *et al.*, 1990) TCC TCC GCT TAT TGA TAT GC ITS5H (Hershkovitz & Zimmer, 1996) GGA AGG AGA AGT CGT AAC AAG G	(modified White *et al.*, 1990) 95°C-15 min 94°C-1 min 57°/60°/63°C-1 min 72°C-1 min 72°C-7 min	2 µM
ETSB-18S ETS	ETSB (Beardsley & Olmstead, 2002) ATAGAGCGCGTGAGTGGTG 18S-ETS (Baldwin & Markos, 1995) ACTTACACATGCATGGCTTAATCT	(modified Beardsley & Olmstead, 2002). 95°C-15 min 94°C-1 min/30 sec 55°/60°C-1 min 72°C-1 min 72°C-7 min	2 µM
GCYC	GCYCFS1 (Smith *et al.*, 2003) AMTGGTTSCTCACTARATC cycR (Möller *et al.*, 1999) ATGAATTTGTGCTGATCCAAAATG	(modified Citerne *et al.*, 2000) 95°C-15 min 94°C-30 sec 50°C-1 min 72°C-1 min	10 µM
rpl32-trnL	rpl32-F (Shaw *et al.*, 2007) CAGTTCCAAAAAAACGTACTTC trnL(UAG) (Shaw *et al.*, 2007) CTGCTTCCTAAGAGCAGCGT	(modified Dong *et al.*, 2012). 95°C-15 min 94°C-3 min 94°C-30 sec 55°C-1 min 72°C-2 min 72°C-5 min	2 µM
TrnL-trnF	trnLc (Taberlet *et al.*, 1991) CGAAATCGGTAGACGCTACG trnLf (Taberlet *et al.*, 1991) ATTTGAACTGGTGACACGAG	(modified Taberlet *et al.*, 1991) 95°C-15 min 94°C-30 sec 63°C-1 min 72°C-1 min 72°C-7 min	10 µM

Table S2. Color palettes to define corolla color in Achimenes species from Mexico (https://colorpalettes.net/).

Color	Color code	Palettes
yellow	#ffdb00	4000
red	#fe0229, #ff0e11, #f32501, #f80039	3863, 3923, 4028, 4037
pink	#f90052, #f3166b	3860, 3997
purple	#cfcef5, #aa93d6, #7f009d, #a18cb8, #a639c2, #d69cf4, #bb89ec	3418, 3424, 3434, 3744, 3908, 4050

Table S3. DNA sequence descriptors for five molecular markers used to reconstruct the phylogeny of the genus *Achimenes*.

	ITS	ETS	GCYC	rpl32-trnL (UAG)	trnL-trnF
N^{o} of base pairs	760	524	608	1117	1032
N^{o} of parsimonious informative sites	116	104	21	83	16
N^{o} of variable sites	239	211	83	226	84
N^{o} of conserved sites	499	295	524	806	862
N^{o} of singletons	120	106	62	137	68
Total number of sequences	25	20	17	19	21

Table S4. Principal component eigenvalues from a phylogenetic PCA of eight floral traits of 21 *Achimenes* species and two outgroups. Calculations were made with natural logarithm transformed variables.

Trait	**PC1**	**PC2**	**PC3**	**PC4**
Corolla length	-0.477	0.604	-0.581	0.231
Corolla width	-0.865	-0.277	-0.309	0.036
Entrance length	-0.933	-0.174	-0.254	0.004
Entrance width	-0.548	-0.788	-0.176	-0.050
Petal length	-0.896	0.087	0.372	0.075
Frontal length	-0.920	0.296	0.117	0.209
Frontal width	-0.953	0.209	0.089	0.188
Corolla base	-0.770	-0.006	0.457	-0.437
Proportional Variance	0.662	0.154	0.112	0.042
Cumulative Variance	0.662	0.816	0.928	0.970
PGLS **PC ~ pollination system**	large bee t = -3.216 P = 0.0045	large bee t = -3.925 P = 0.0009 small bee t = -7.048 P = 0.00001	hummingbird t = -4.957 P = 0.0001 large bee t = -3.156 P = 0.0052 small bee t = -1.963 P = 0.0645	Not statistically significant

Table S5. Transition models of three discrete floral characters of *Achimenes* species and three outgroups.

geiger:: fitDiscrete Models	**Pollination system**	**Corolla color**	**Corolla shape**
	AICc	AICc	AICc
Equal rates	65.96	76.22	51.38
Symmetric	77.19	105.02	52.91
All rates different	114.78	520	61.51

Table S6. Evolution models of corolla width and entrance length of *Achimenes* species and two Gesneriaceae species as outgroups.

geiger::fitContinuous models	**Corolla width Ln(mm) AICc**	**Corolla length Ln(mm) AICc**
BM	42.09	38.23
EB	45.05	41.18
OU	39.88	38.17
trend	43.61	40.10
lambda	39.67	41.18
white noise	39.82	38.23
OUch:: Hansen models		
Brownian motion model	46.66	40.56
“global” (one regime for all branches)	37.88	34.33
BayesTraits’ pollinator reconstruction nodes	24.39	20.88
“Small bee”	22.38	23.59

Table S7. Phylogenetic signal of individual log-transformed floral traits of *Achimenes* genus.

Floral trait	**Blomberg's K**	**P**
Corolla length	0.279	0.008
Corolla width	0.212	0.079
Entrance length	0.190	0.115
Entrance width	0.242	0.029
Petal length	0.096	0.887
Frontal length	0.133	0.465
Frontal width	0.122	0.613
Corolla base	0.133	0.483

Table S8. Models of multivariate evolution of five floral traits: corolla width, corolla length, entrance width, petal length and frontal width.

Model	**AICc**	**Number of parameters**
OUM1	148.17	35
OUMM	149.25	50
BM same rates	190.67	16
BMM proportional	197.57	23

BM1 not covariance	264.20	10

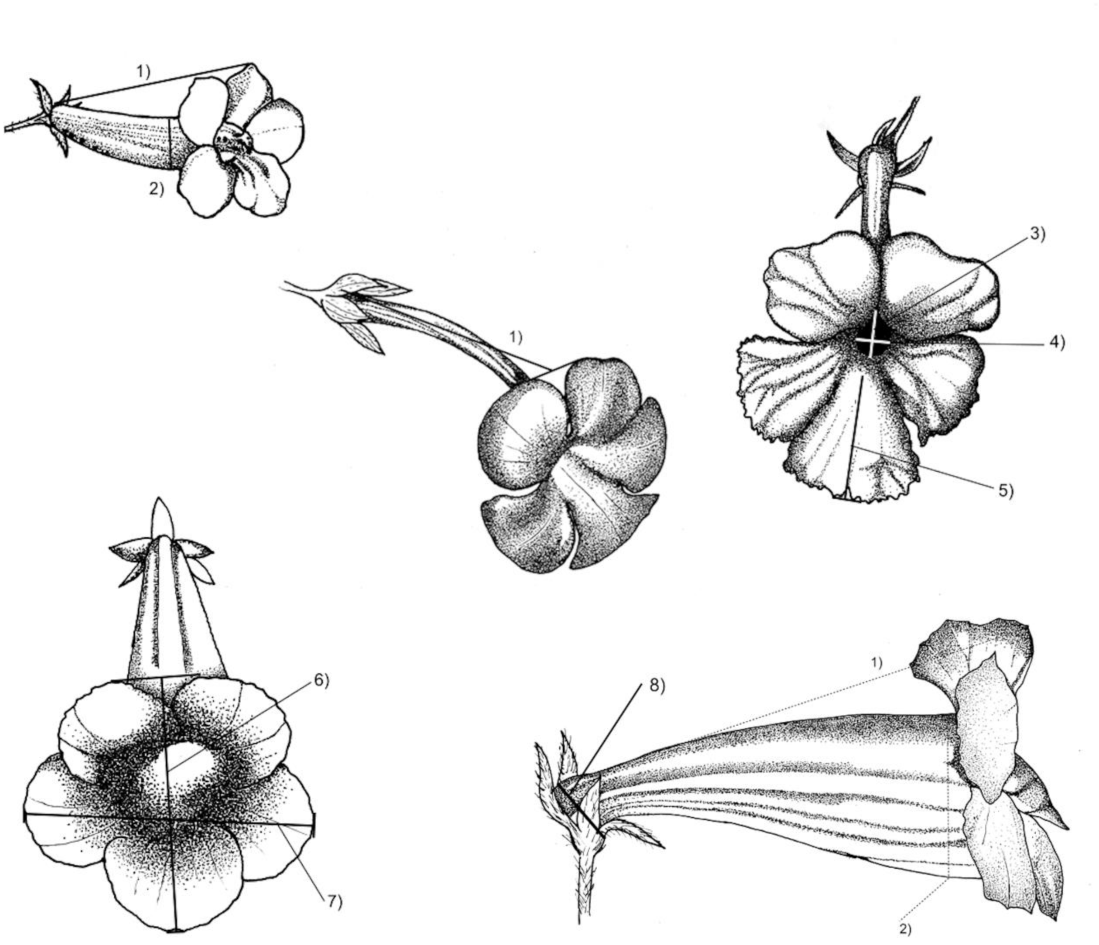

Figure S1. Floral morphological traits measured in 21 *Achimenes* species and in *Moussonia* and *Eucodonia* outgroups. 1) Corolla length, 2) corolla width, 3) Corolla length at mouth, 4) corolla width at mouth, 5) lower petal length, 6) frontal length, 7) frontal width, 8) corolla base.

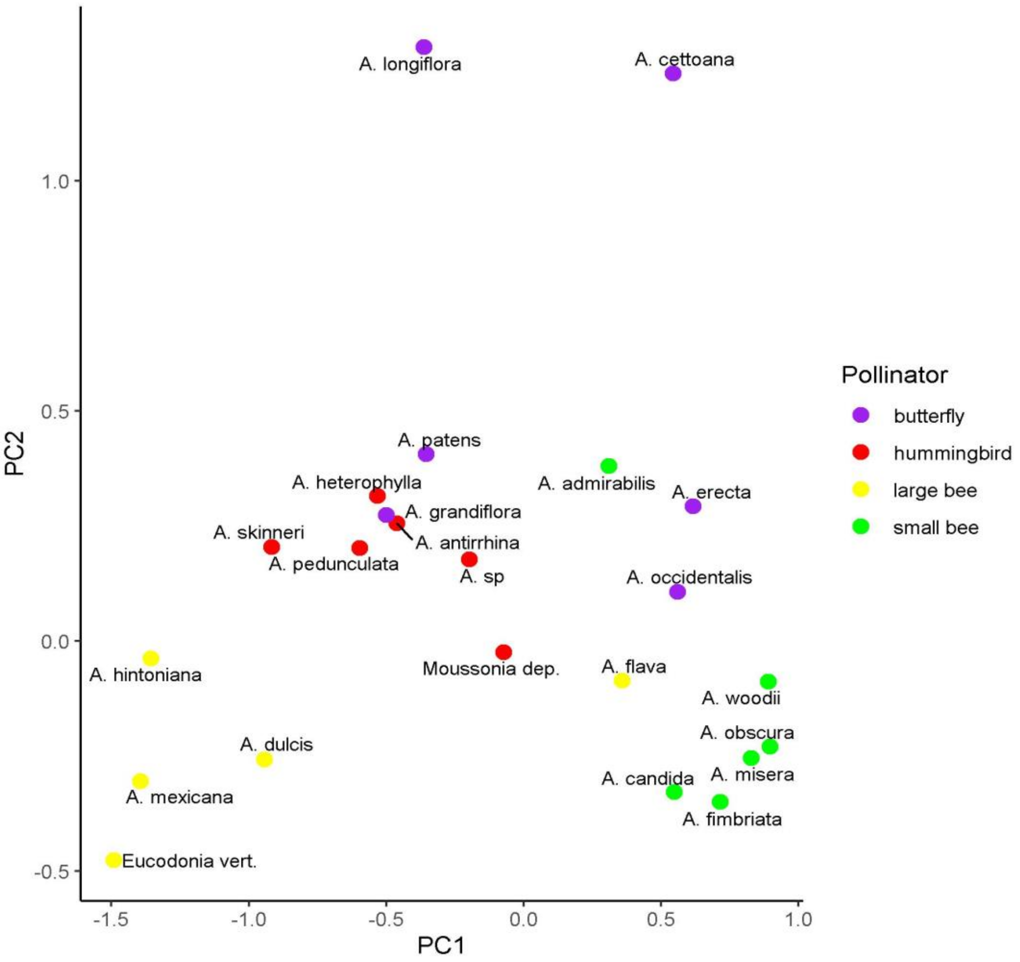

Figure S2. A) Phylogenetic principal component analysis of eight floral morphometric traits (natural logarithm transformed). Species include 21 ingroup species of *Achimenes* and two Gesneriaceae outgroups.

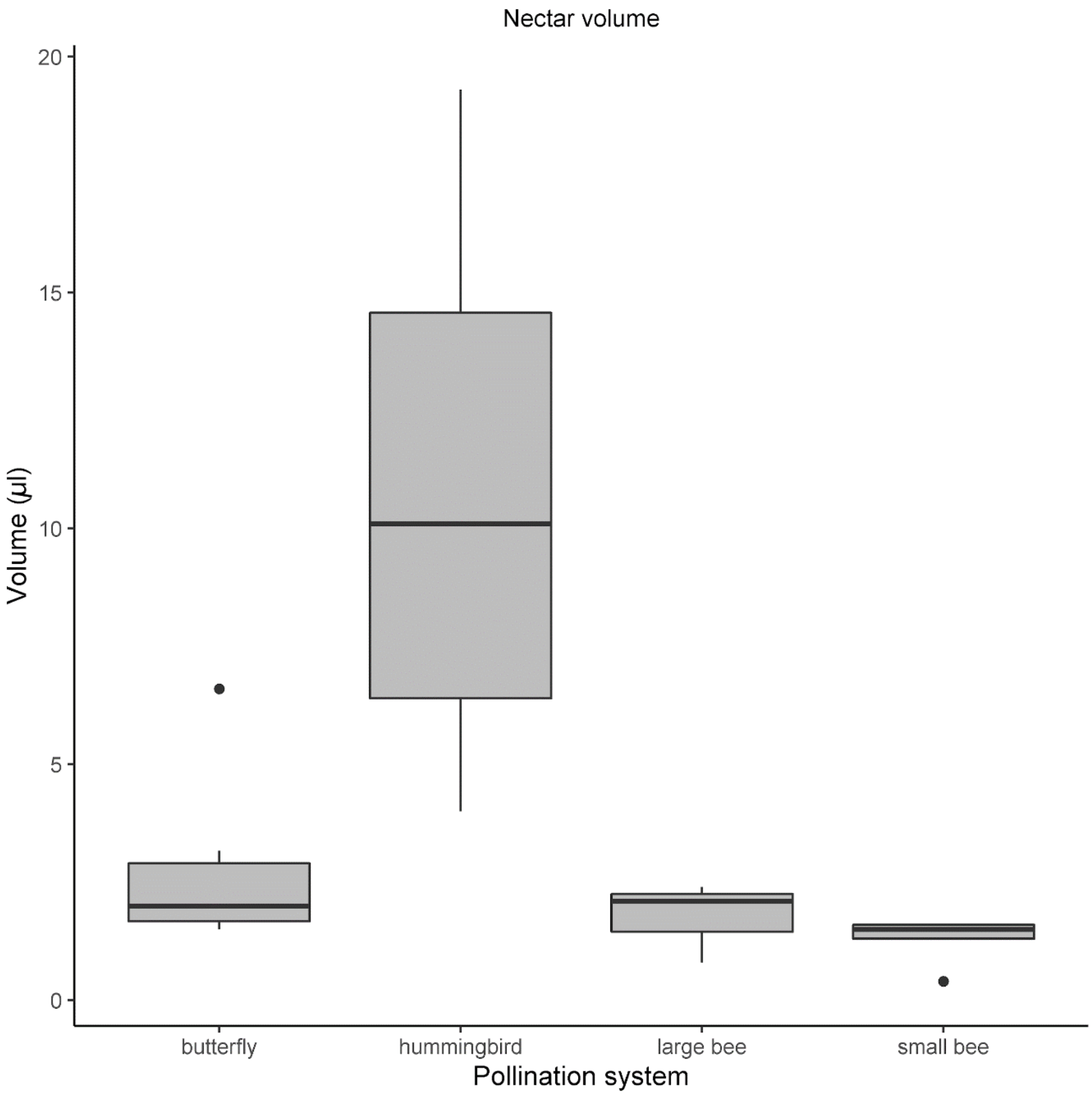

Figure S3. Nectar production contrasted between four pollination systems in 18 *Achimenes* species from Mexico. Nectar volume was measured in greenhouse specimens at UNAM, Campus Morelia.

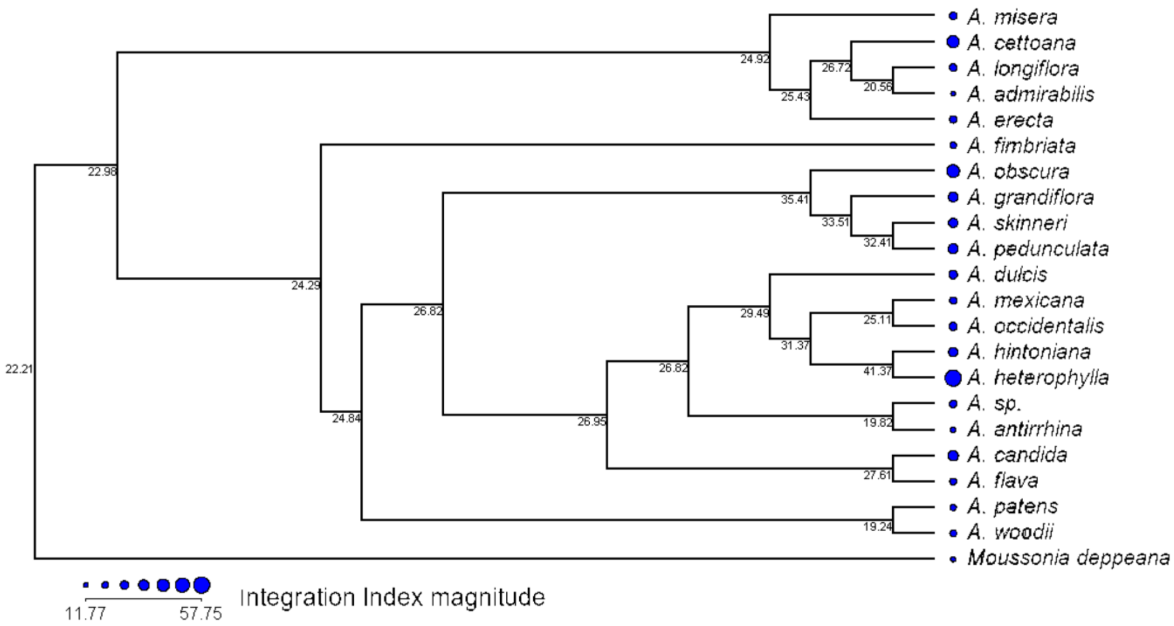

Figure S4. Floral integration mapping on phylogeny of 21 *Achimenes* species and one outgroup. Small numbers below branches are reconstructed floral integration indices. Blue dots are extant floral integrations relative to circle size.

CAPÍTULO III. THE ROLE OF BIOTIC INTERACIONS IN THE EVOLUTION OF FLORAL DIVERSITY AND BREEDING SYSTEMS IN THE FAMILY GESNERIACEAE: A GLOBAL REVIEW.

Running title: Current knowledge of the Gesneriaceae

Title: The role of biotic interactions in the evolution of floral diversity and breeding systems in the family Gesneriaceae: a global review.

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RESUMEN

La familia Gesneriaceae es una familia de angiospermas que comprende 3500 especies alrededor del mundo. La diversidad de especies es reflejada en la variación de los caracteres florales y vegetativos. La diversidad floral es explotada ornametalmente, pero, tanto la historia natural, como los análisis comparativos son necesarios para entender los procesos involucrados en la diversificación floral y los patrones de evolución floral en el grupo. Este estudio es una revision de la información disponible de la ecología reproductiva y evolución de la familia Gesneriaceae. Encontramos que la selección mediada por los polinizadores es una de las explicaciones más importantes para la diversificación floral en la familia Gesneriaceae, sin embargo, pocos estudios toman enfoques tanto cuantitativos, como experimentales para medir la selección sobre los caracteres florales, o pocos utilizan análisis comparativos para descubrir factores potenciales que dirigen la diversificación floral.

Abstract

The Gesneriaceae is a diverse angiosperm family with around 3500 species worldwide. Species diversity is also mirrored by variation in floral and vegetative characters. Floral diversity is exploited ornamentally, but natural history and comparative analyses are needed to understand the processes involved in floral diversification and the patterns of floral evolution of the group. This study is a literature review of the information available on the reproductive ecology and evolution of the family Gesneriaceae. We found that pollinator-mediated selection is proposed as one of the main explanations for floral diversification in Gesneriaceae, but few studies take quantitative or experimental approaches to measure selection on floral traits, or comparative analyses to uncover potential factors driving floral diversification.

INTRODUCTION

The family Gesneriaceae is a large and diverse plant family that comprises more than 3500 species distributed in about 150 genera worldwide (Weber, 2004). The subfamilies Cyrtandroideae and Epithemateae are distributed in Europe, Africa and Asia and are called “Old World” subfamilies, with representations in both tropical and temperate regions. The American or “New World¨ subfamily includes the Gesnerioideae, and the Coronanthereae that encompasses both Oceania and South America. Gesneriaceae subfamilies are typical of tropical and subtropical environments but with center of diversity in tropical regions of America and Asia (e.g. Perret *et al.*, 2007; Clark *et al.*, 2009; Roalson & Roberts, 2016). Most Gesneriaceae species inhabit continental regions, but there have been dispersals to oceanic islands with two radiations in the Hawaiian and Antillean archipelagos (Clark *et al.*, 2008, 2009; Martén-Rodríguez *et al.*, 2010). Diversification both in mainland and insular environments has resulted in a diversity of life forms, as well as in an extraordinary floral diversification. A comprehensive understanding of the factors underlying diversification requires information on natural history, ecology, genetics and systematics of the species of different plant groups. The ecology and evolution of flowers and their interactions appears to be key to understanding some of the large radiations that have occurred in the evolution of Gesneriaceae.

The evolution of floral diversity has been attributed to selective forces exerted by mutualistic and antagonistic plant-animal interactions, as well as the abiotic environment (Kay *et al.*, 2006; Strauss & Whittall, 2006). Floral diversity in the Gesneriaceae is depicted in different pollination syndromes (i.e. groups of floral characters associated with particular pollinators, Faegri & van der Pijl, 1979). Syndromes generally represent floral phenotypes adapted to the most effective pollinator functional groups, and they do necessarily imply ecological specialization (i.e. one or few pollinator species; Stebbins, 1970; Fenster *et al.*, 2004; Rosas-Guerrero *et al.*, 2014). Assessments of pollination syndromes and level of specialization have only been conducted in a few lineages within the Gesneriaceae (e.g. SanMartin-Gajardo *et al.*, 2004, 2005b, Martén-Rodríguez *et al.*, 2009, 2015).

Specialized floral traits are present in both cross and self-fertilized systems (Barrett, 1998), and they often reflect selection to ensure reproduction in environments where pollinator visitation is low or variable (Fenster & Martén-Rodríguez, 2007). Many Gesneriaceae species depend on pollinators as the most important mechanism to breed, but others use autonomous self-pollination as a reproductive assurance mechanism (Sanmartin-Gajardo & Sazima, 2005b; Martén-Rodríguez & Fenster, 2010; Ramírez-Aguirre *et al.*, 2016). Recently, phylogenetic and comparative methods have been used to infer the adaptive function of floral characters mediated by pollinators (Smith *et al.*, 2010). Molecular phylogenetics and comparative biology have been useful to infer patterns of floral trait evolution in various Gesneriaceae clades (Perret *et al.*, 2001; Martén-Rodríguez *et al.*, 2010; Clark *et al.*, 2015; Roalson & Roberts, 2016); however, there are many interesting Gesneriaceae groups that warrant further study.

In this study, we conducted a search of the available information on the floral biology, plant reproductive systems, and evolutionary studies of the family Gesneriaceae. The specific goals of the study were to: (1) evaluate the association between floral traits, such as corolla colour, shape rewards and attractants and the described pollinators, (2) assess the occurrence of different breeding systems in relation to pollination systems, (3) synthesize results from comparative phylogenetic studies on floral evolution This synthesis of the available literature on the reproductive ecology and evolution of the Gesneriaceae will hopefully indicate knowledge gaps and lines of research that require attention in future studies.

Methods

In order to compile the current knowledge on the reproductive biology and floral evolution of the family Gesneriaceae, we conducted a search on the ISI Web of Science from 1900 to 2019, using the following keywords: “gesneriaceae” AND “reproduct” AND “pollinat*”. We also searched for articles that included phylogenetic approaches encompassing floral trait evolution associated with pollinator system, with “gesneriaceae” AND “phylogen*” key words. We used articles that included at least one of the following aspects: pollinators observed in the field, floral traits and rewards quantified in the field, breeding systems documented in the field or greenhouse, and comparative studies of floral evolution. We also reviewed publications cited in these articles, which some publications are in specialized journals, like Selbyana or Baileya, which did not appear in the main search. For each species, we recorded the following variables according to the available information: life form, floral shape, morphology, colour, reward, number of pollinator species observed, pollinator identity, fruit or seed set under different pollination treatments (apomixis, autonomous self-pollination, hand-self pollination, outcross hand-pollination, natural pollination). When data for a species was different between reports, we kept both records, otherwise we combined it. An additional search was done with “gesneriaceae” AND “nectar” key words, because nectar is the most frequent reward in angiosperms (Endress, 1994). We also reviewed all phylogenetic studies that evaluated the evolution of floral traits and/or pollination systems.

Results and discussion

We found 34 published articles and our own unpublished data reporting results on floral biology (i.e. studies that report pollinators, reproductive systems, rewards, pollination syndromes) studies conducted in the field. A total of 218 different species were reported both in continental populations (156), on islands (51), and in both (2); one had missing localities (Dressler, 1968b). Fifty three mainland and 12 insular species had missing data on floral visitors or pollinators. Of the species with available data, 110 were from mainland regions while 42 were from islands. The pollination systems of these species included bees, bats, and hummingbirds across geographic regions while moth pollination was exclusively reported on islands (Figures 1 and 2). The latter result may reflect the limited number of studies reporting nocturnal pollination observations in mainland regions.

For nectar production we found 20 articles in the main search and three additional studies in the specific search. Of the total number of nectar reporting articles, 16 reported nectar volume. All articles were included to the final database.

In terms of comparative studies conducted within a phylogenetic framework, we found 16 articles that evaluated different questions concerning the evolution of floral traits (e.g. floral morphology, corolla colour, corolla shape, nectar traits, and pollen presentation) and pollination systems (Harrison *et al.*, 1999; Perret *et al.*, 2003; Roalson *et al.*, 2003; Hughes *et al.*, 2006; Clark *et al.*, 2006; Perret *et al.*, 2007; Perret *et al.*, 2011; Martén-Rodríguez *et al.*, 2010; Wang *et al.*, 2010; Clark *et al.*, 2015; Roalson & Roberts, 2016; Shulte *et al.*, 2015; Serrano-Serrano *et al.*, 2015, 2017). Pollination syndromes were also evaluated or mentioned in relation to previously documented pollinators.

Pollination systems

The most common pollinator groups were birds, bees, bats, moths and butterflies in decreasing order. Nemestrinidae flies (Diptera:Nemestrinidae) have been reported in African Gesneriaceae (Potgieter & Edwards, 2005) and hawkmoths were allegedly reported as pollinators of Cyrtandra species (Gardener & Daehler, 2006). Most species had specialized pollination systems (i.e. one pollinator functional group), but a considerable number of species had mixed pollination systems with more than one pollinator functional group (Table 1). Mixed pollination systems were more common on islands (29% of insular species), while specialized pollination systems were predominant in mainland regions (Figure 1). This result suggests that unpredictable pollinator visitation and low diversity of pollinator functional groups may promote transitions from specialized to mixed-pollination systems-as suggested for Antillean Gesneriaceae (Martén-Rodríguez *et al.* 2010). On islands plant diversification and speciation may also occur by enforcement of reproductive isolation mechanisms or by hybridization (e.g., *Cyrtandra*, Johnson *et al.*, 2015; Johnson *et al.*, 2019).

When grouping specialized pollination systems by their presence in mainland or island regions, we found that hummingbird and bee pollination systems predominate in the mainland, although with various other animal groups also act as pollinators (bats, flies, butterflies, Euglossini-bees, mixed). On islands, different pollination systems were more evenly represented including bees, bats, hummingbirds, moths and mixed-pollination systems (Figure 2). Our current knowledge supports that there are more pollinator-specialized species in mainland habitats, in contrast with more pollinator-generalized species on islands; this difference may be related with differences in pollinator abundance and diversity, since pollinator groups are less diverse, less frequent and less reliable visitors on islands (Martén-Rodríguez *et al.*, 2015). In this context we might ask, what factors underlie differences in pollinator diversity among different regions or habitats? Both abiotic and biotic factors influence pollinator diversity; for example, the diversification of hummingbirds in the Antilles was associated with local conditions, such as topography or humidity (Dalsgaard *et al.*, 2018); while competition for floral resources was proposed as a major factor maintaining different hummingbird assemblages. Whether this trend is true for bats, bees and other birds that are pollinators of Gesneriaceae species remains less understood. Alternatively, mainland taxa with small and scattered populations constrained to particular habitats, may be diverse due to competition among local pollinator faunas.

Among birds visiting flowers, hummingbirds were the most commonly reported pollinators, suggesting that hummingbirds must play an important role in the diversification of Neotropical Gesnerioideae (Serrano-Serrano *et al.*, 2017). Bananaquits were also observed visiting some insular species of New World Gesneriaceae. In the Old World, birds like honeyeaters and sunbirds were reported as pollinators mainly of *Aeschynanthus* and *Rhabdothamnus* species. Floral morphology of both New World and Old World bird pollinated species is similar, for example, American *Columnea* and Asian *Aeschynanthus* flowers share brightly colored, tubular corollas, suggesting convergence in floral traits associated with bird pollination. Overall, ornithophilous species have mainly red, orange and yellow corollas (or a combination of colours), tubular flowers and zygomorphic (bilaterally symmetric) corollas (Figures 3 and 4). Thus, hummingbirds, honeyeaters and sunbirds, probably exert similar selection pressures on floral traits such as corolla shape and colour. In contrast, bananaquits are only pollinators of Gesneriaceae species on islands, where they generally visit legitimately flowers with sub campanulate corollas, also pollinated by bats (Martén-Rodríguez *et al.*, 2009).

Bees are a diverse group of pollinators (Michener, 2007). In New World Gesneriaceae, Euglossini and Anthophorini were documented collecting nectar, pollen or fragrances from flowers. In Old World species, most of the pollinators were bees belonging to Anthophoridae (Apidae: Anthophorini) and *Bombus* (Apidae: Bombini). Bee pollinated Gesneriaceae species share similarities in flower colour and corolla shape both in New World and in Old World. Since visual abilities related to color preferences in bees are thought to have evolved earlier than flowers, convergence in flowers color among bee pollinates lineages may reflect pre-existing bias in sensory abilities of bees (Schiestl & Johnson 2013). The most common colors of bee flowers were lilac, purplish, white, yellow or a combination of those colours (Figure 3). Many species are zygomorphic, however there are actinomorphic (or radially symmetric) flowers both in the Old and New World. Actinomorphic species fit the *solanoid* syndrome (i.e. morphologically similar to *Solanum* (Solanaceae) flowers sensu Faegri, 1986), usually lacking nectaries, with pollen and possibly fragrances offered as rewards. *Solanoid* syndrome genera include *Phinaea*, *Niphaea*, *Amallophylum*, *Napeanthus*, *Conandron*, *Tengia* and *Thamnocharis*.

A very specialized type of melittophily is the buzz pollination syndrome characterized by poricidal anther dehiscence, actinomorphic flowers and scarce or absent nectar. The

Antillean genus *Bellonia* (New World) fits the buzz-pollination syndrome (Martén-Rodríguez *et al.*, 2009). The Old World genera *Ramonda*, *Paraboea*, *Saintpaulia* have connate anthers and anther dehiscence in the form of a slit. In these species, large bees must probably vibrate their bodies to release pollen as a reward (Weber, 2004).

Bats were reported as pollinators of five species, all of them from the New World. Accordingly, there are no Gesneriaceae species with bat pollination syndrome in Africa, Asia or Oceania. Species with chiropterophilous syndrome have pale corolla colours, such as green, white or yellow, and wide and campanuloid forms, with a strong odour, nocturnal anthesis, and nectar as the main reward (Table 1). Bat pollination is only represented in New World species by Microchiroptera bats, specifically members of the Phyllostomideae family. It is possible that both genetic and developmental routes in Old World Gesneriaceae constrain changes in floral traits, but these have not been documented. Another possibility is that Old World megachiropteran species foraging behaviour is different from that of New World species, and that there is more floral diversity in the New World than in the Old World because of differences in orogeny (Fleming *et al.*, 2009). The same authors also suggest that hovering ability of New World bats could have. Interestingly, Baker *et al.*, (1998) found that nectar sucrose composition was different between New World and Old World bat-pollinated species of various families. Alternatively, bat-pollination in Gesneriaceae species may be related to woody habits and examples of woody plants in seasonal habitats and bat-pollinated can be found elsewhere (e.g. Heithaus *et al.*, 1975; Sazima *et al.*, 1999). Woody plants and, specifically their root system, may be strategy to allocate more resources to reproductive function (and other functions, such as vegetative growth or defense) (Pregitzer, 2003), thus allowing a bigger floral display and its consequent nectar quantity, however this has not been tested yet in Gesneriaceae.

Floral traits

Corolla colour and shape

In the Gesneriaceae, corolla colour is highly variable among species, but predominant colours are red, yellow, purple and white (Figure 3). In particular, there are tight associations between corolla colour and pollination systems in *Gesneria*, *Sinningia*, *Paliavana*, *Rhytidophyllum*, *Achimenes*, *Drymonia*, *Nematanthus* and *Rhabdothamnus* species (Buzato *et al.*, 2000; SanMartin-Gajardo & Sazima, 2004; Sanmartin-Gajardo & Sazima, 2005b; Martén-Rodríguez *et al.*, 2009; Sanmartin-Gajardo & Vianna, 2010; Anderson *et al*., 2011; Martén-Rodríguez *et al.*, 2015; Clark *et al.*, 2015; Ramírez-Aguirre *et al.*, 2016). Colours associated with pollinators in this species are red for hummingbirds, green for bats, white or lilac for bees and purple for butterflies. Other genera, like *Columnea*, *Besleria*, *Aeschynanthus*, *Seemania* species have corolla colours that fit the classical definition of ornithophily, i.e. reddish colours, (Camargo *et al.*, 2011; Rahman, 2011; Stein & Hensen, 2013; Shulte *et al.*, 2015). Forty two species from both New and Old World fit the melittophily syndrome definition (Dressler, 1968b; Wiehler, 1976; Kato *et al.*, 1989, 1993; SanMartin-Gajardo & Sazima, 2004; Wang, *et al.*, 2004; Hughes *et al.*, 2006, 2007; Pu *et al.*, 2009; Tang *et al.*, 2009; Wang *et al.*, 2011; Bogacheva *et al.*, 2013; Bogacheva-Milkoteva *et al.*, 2013; Guo *et al.*, 2013; Guo & Wang, 2014; Martén-Rodríguez *et al.*, 2015; Ling *et al.*, 2017). Only five species of Gesneriaceae in genus *Achimenes* were reported as butterfly-pollinated, and from them, four were reported as purple, one was red and one was white coloured (Ramírez-Aguirre *et al.* 2019, unpub. data).

Some corolla colours had a higher representation among pollination systems (represented as pollinator functional groups) than others. For bat-pollinated species light-green and yellow-cream (from now on, cream in figures) corollas accounted for 60% of the records, while for hummingbird-pollinated species, red corollas represented 51% of the records (Figure 3). For moth-pollinated species only white and yellow-cream colours were represented. Interestingly, for bee-pollinated species there was a high variation in corolla colour, with white and lilac corollas mainly represented (Figure 3).

Foraging pollinators use different clues to find floral resources with their sensory abilities (i.e. vision, smell, touch; Chittka & Waser, 1997; Chittka & Thomson, 2004; Schiestl & Johnson, 2013). In turn, the use of floral rewards enhances pollinator visitation and plant fitness (Mitchell, 1993; Meléndez-Ackerman & Campbell, 1998; Medel *et al.*, 2003). Together, our results suggest that corolla colour defined by humane eye is a proxy to define pollinator visual attraction Gesneriaceae floral colour. Apart from humane visual perception, UV floral reflectance is well perceived by insects, such as bees, so patterns of floral UV reflectance are interpreted as nectar guides or cues to insects that enhance pollinator (Jones & Buchmann, 1974; Utech and Kawano 1975; and in some birds, Bennett & Cuthill, 1994). However, there are not any papers that documented UV reflection patterns in addition to human-vision colours in Gesneriaceae, leading to incompleteness of floral colour evolution.

Corolla shape is related to the mechanical fit between pollinators and flowers by facilitating access to reward and changing pollen-deposition sites on pollinators, so it is considered a pollination efficiency trait (Grant, 1994; Castellanos *et al.*, 2004; Rosas-Guerrero *et al.*, 2011). Records of corolla shape are represented more frequently in certain pollinators than in others, for example more than 50% of tubular corollas appear in hummingbird pollinated species (Figure 4). Others, such as campanulate corollas are widely represented in bat-pollinated species. For moth-pollinated species, tubular, campanulate and urceolate were found in equal frequencies, while for bee-pollinated species tubular and campanulate corollas represented about 80% of the records (Figure 4).

Vegetative traits and their pollination related functions

Floral calyces generally have the function of protecting flower buds from biotic or abiotic damage; however, in some plant lineages, they may have a role in pollinator attraction. In the Gesneriaceae genera *Columnea*, *Drymonia*, *Alloplectus, Nemathanthus,* brightly coloured calyces are often associated with hummingbird pollination with red, orange, yellow and pink colours (Figure 5). These coloured calyces may be the main visual attractive structure, in species with lighter corolla colours (e.g. *Nematanthus brasiliensis*), they might provide contrast to the flower attraction unit, when calyx and corolla have bright but different colours (e.g. *Drymonia strigosa*), or they might potentially increase the visual attraction field if calyx and corolla are the same colour (e.g. *Gasteranthus otongensis*); nevertheless, the attractive function of coloured calyces remains untested. Another possible case of attraction of non-floral structures are the leaves of *Columnea florida* species. Leaves have red spots in the tip, on the abaxial side of the leaf. It has been proposed that, since flowers are small and inconspicuous, large coloured leaves may serve as a visual clue for hummingbirds (Jones & Vickers, 1972); however, this idea has not been formally tested. Interestingly, all the mentioned genera are epiphytes and many are hummingbird-pollinated, so brightly coloured calyces may be an important trait to advertise birds inside green canopies. Epiphytes are usually pollinator-specialized, with bird-, bee- or bat-pollination systems and are mainly out-crossing, suggesting high dependence on pollinators

(Bush and Beach, 1995; Ramírez-Aguirre *et al.*, 2016; Martén-Rodríguez *et al.*, 2015), which would indicate that visual clues are important in attraction at least to birds to ensure pollinator visitation.

Other functions of flower traits

Floral traits may have other functions in addition to pollinator attraction. For instance, floral parts may be subject to high levels of herbivory; thus, floral traits may reflect selection to reduce the consumption or damage of the flower's reproductive structures (Strauss & Whittall, 2006). In the Gesneriaceae, this has been reported for *Chrysothemis friedrichsthaliana*, which has calyx trichomes that produce a liquid that fills the calyx and protects buds from moth-lepidopteran herbivory (Carlson & Harms, 2007). However, as far are aware, there is not any other Gesneriaceae species with floral traits that also represent an adaptation to deter herbivores, but it is possible that different floral volatiles such as naphthalene serve as deterrents (Azuma *et al.*, 1996), or the presence of secondary metabolites in petal pubescence might prevent herbivory in Gesneriaceae but until now, both these traits and functions are hypothetical.

Rewards

Floral rewards, such as nectar, directly influence pollinator visitation, by setting sexual advertisement or energetic resources that stimulate pollinators'sexual attraction or satisfy pollinators'nutritional preferences (Schiestl *et al.*, 1999; Johnson & Nicolson, 2008; Schiestl, 2015; Tiedge & Lohaus, 2017). In turn, rewards such as floral nectar, affects pollinator visitation and plant fitness (Mitchell, 1993; Schemske & Bradshaw, 1999). Nectar is one of the most important rewards that plants offer to pollinators (Endress, 1994). Accordingly, for Gesneriaceae species, nectar was the main reward reported, but pollen, oils and fragrances were reported as floral rewards too (Table 3). There were also different patterns of nectar production associated with pollinators. Nectar volumes were highest for most bat pollinated species, while lower volumes were recorded for hummingbird and bee pollinated species (Table 3). However, some bee pollinated species produced a higher volume of concentrated nectar, a lack of correspondence with the classical mellittophilous syndrome (Figure 3). The combination of nectar traits, such as high volume and high sugar concentration, may be to satiate large bees that feed from them (Steiner, 1985; Ramírez-Aguirre *et al.*, 2016). Nectar sugar concentration was variable for Gesneriaceae species recorded. Most bat pollinated species had higher sugar concentration or sugar content, hummingbird pollinated species had lower to moderate sugar contents and bee pollinated species present variable sugar in nectar (Table 4). Furthermore, sugar concentration in Sinningieae was found to be similar between bee and hummingbird syndromes and authors suggest that nectar sugar composition may be phylogenetically constrained to change (Perret *et al.*, 2001). Apart from possible phylogenetic constraints due to genetic or developmental restrictions in nectar production, pollinator preference may be a cause of nectar production variability. For example, both hummingbirds and sunbirds showed no preference in sucrose or hexose solutions as far as they provide the same amount of caloric income when diluted (Fleming *et al.*, 2004).

There were non-typical nectar volumes and sugar concentration in the bee pollinated species *Drymonia serrulata* and *D. oinochophylla* (Steiner, 1985; Ramírez-Aguirre *et al.*, 2016). It is possible that high nectar volumes are related to large bee pollination, since *D. oinochrophylla* and *D. serrulata* are pollinated by Anthophoridae and Euglossine bees. Alternatively, the allometric relation between flower size and nectary size, could result in higher nectar volumes for larger flowers, and not due to pollinators (Galetto & Bernardello, 2004). Both *Drymonia* species are relatively large flowered and allometric relations may favour large nectar volumes associated with large glandulous nectaries. In order to discard an allometric relation among flower size and nectar production, standardizations on size should be made to control for floral size with more species and in a phylogenetic context. A better definition for nectar composition is needed for pollinator syndrome classical descriptions in order to understand pollinator mediated selection in this trait, because pollinators have different energetic demands and foraging behaviour (e.g**.** Feinsinger & Colwell, 1978; Ohashi & Thomson, 2009). Unfortunately, there is a lack of information of nectar production in Gesneriaceae, and its relation with pollinator systems is less understood. The genera *Sinningia*, *Paliavana*, *Gesneria*, and *Rhytidophyllum* are the most completely described for nectar volume and nectar sugar concentration, although there are scattered reports for nectar production of other Gesneriaceae species (Roelofs, 1979; Ayala, 1986; Feinsinger *et al.*, 1986; Freeman *et al.*, 1991; Stiles & Freeman, 1993; Kato *et al.*, 1993; Bernardello *et al.*, 1994; Sanmartin-Gajardo & Freitas, 1999; Buzato *et al.*, 2000; Perret *et al.*, 2001; Sanmartin-Gajardo & Sazima 2005b; Ornelas & Ordano, 2007; de Ávila, 2010; Martén-Rodríguez & Fenster, 2008; Ferreira & Viana, 2010; Ramírez-Aguirre *et al*., 2016). Thus, finer conclusions about the relation between nectar production and pollinator systems may be biased because of phylogenetic relationships and underrepresentation of other species. However**,** based on trends in other genera and the findings in Gesneriaceae, some conclusions can be drawn: 1) nectar volumes for bat species are higher than in any other syndrome (except of bee pollinated *Drymonia oinochrophylla*), 2) when reported, nectar concentration (expressed as °Brix) in hummingbird-pollinated species was more concentrated than in bat-pollinated species and lower than bee-pollinated species (Figure 3).

Pollen is the second offered floral reward (Simpson & Neff, 1981). Pollen as a reward, is associated with pollen collecting bees, like *Bombus* species, Anthophoridae and female Euglossine bees and Meliponids. In Gesneriaceae, pollen-only rewarding species were *Janakea heldrechii*, *Bellonia aspera*, *Paraboea rufescens*, *Saintapulia teintensis* (and probably most *Saintpaulia* species), *Phinaea*, *Niphaea*, *Amallophyllon* (Vokou *et al.*, 1990; Gao *et al.*, 2006; Boggan *et al.*, 2008; Martins, 2008; Martén-Rodríguez *et al.*, 2015). All the Gesneriaceae species that offer pollen as the main and only reward are closely or completely actinomorphic, in particular *Phinaea*, *Amallophyllon*, *Bellonia aspera* and *Niphaea* fit the *solanoid* flower type and are pollinated by vibrating bees (Faegri, 1986).

Other floral rewards: fragrances and oils

Floral scents promote attraction to pollinators and thus promote out-crossing in plants with specialized pollination (Raguso, 2008). In Gesneriaceae family, fragrances and oils are associated with pollination by Euglossine male bees' that collect fragrances to attract female bees. Fragrances are offered by some species of *Drymonia* and by *Gloxinia perennis* (Dressler, 1968a and 1968b; Steiner, 1985; Martel *et al.*, 2019). *Gloxinia perennis* individuals emit sweet fragrances similar in composition with the ones found in orchid genera, such as *Catasetum* (Vogel, 1966; Martel *et al.*, 2019). Volatile emission takes place during day and stops during night, and Euglossine bees are the most effective and abundant pollinator in these species (Martel *et al.*, 2019).

There is another interesting case where fragrances are related to pollinators' foraging schedules. Individuals of the species *Paliavana prasinata* and *Sinningia brasiliensis* emit strong fruity odors during evenings and through the night when *Glossophaga soricina* and *Anoura caudifer* (Phyllostomidae) visit, while *Paliavana tenuiflora* with a mixed syndrome (hummingbird and bat syndromes) emits odours during the two- night anthesis and it is not pollinated by bats but by hummingbirds (Sanmartin-Gajardo & Sazima, 2005a).

Few other species are described as fragrant by horticulturists (e.g. *Achimenes dulci*s, *Chiritopsis repanda var. guilinensis, Codonathe, Nautilocalyx punctatus*) (Boggan, 1996). Interestingly, floral morphology of *Achimenes dulcis* and *Nautilocalyx punctatus* species fits the gynandro-euglossophilous syndrome and the melittophilous syndrome (Wiehler, 1976a). If gynandro-euglossophilous flowers are effectively pollinated by large Euglossine or Anthophoridae bees then, independent origins for the production of fragrance rewards are mediated by selection of fragrance collecting-bees

Another possible effect of large bee selection is the presence of oils as floral rewards. Oils may be a nutritive source of lipids or may have a nesting-glue or nesting-antiseptic purpose and large bees have been seen to collect them actively, however other functions than being part of some volatiles have not proved yet (Simpson & Neff, 1981). In Gesneriaceae, oils produced by *D. serrulata* aid to stick pollen grains to *Epicharis* (Centridini) and *Euglossa* (Euglossini) bee species while they forage for nectar (Steiner, 1985). The Gesneriaceae family shows that independent evolution of other attraction traits rather than nectar, and a possible evolution of special rewards has appeared in angiosperms. However, for Gesneriaceae, little is known for the evolution of neither fragrances nor oils as floral rewards.

Mating and Breeding systems

We found 37 studies that evaluate breeding systems through experimental hand pollination. The outcomes of both pollinations indicate whether pollinator is required to form fruits or seeds (outcrossing species) or not (selfing species). In our literature search all species are self-compatible (ie. production of seeds with self-pollen), with self-compatibility indexes

(SCI; hand self-pollination/hand cross-pollination) ranging from 0.18 to1. Most Gesneriaceae species, independently of their phylogenetic position are self-compatible, and only two species, *Mitraria coccinea* and *Asteranthera ovata* (insular species pollinated by hummingbirds), were continuously reported as self-incompatible (Smith-Ramírez *et al.*, 2005). Riveros *et al.* (1996) described *Mitraria coccinea* as self-compatible with a self-incompatibility index of 0.52 and *Astheranthera ovata*, has a self-compatibility index of 0.18. There is some debate due to the definition of self-compatibility in angiosperms (e.g. Raduski *et al.*, 2011).We think that invoking self-incompatibility in a species close to 0.2 SCI, in one population at one time, may be risky, since there is variability in space and time within and between populations (Ferrer & Good-Avila, 2007; Whitehead *et al.*, 2018). Overall, results in our search suggest that Gesneriaceae species are mainly outcrossing (Table 5).

However, the capacity for autonomous fertilization was present in most species and represented as self-fertility index (SFI, autonomous pollination/ hand cross-pollination) (Table 5). Self-fertilization due to autonomous-pollination has been proposed as a reproductive assurance mechanism (Fenster & Martén-Rodríguez, 2007). Martén-Rodríguez *et al.* (2015) showed that self-fertilization in Neotropical Gesneriaceae is present in specialized pollination systems in both insular and mainland habitats, probably due to variations in pollinator visitation. The same authors found an interesting association of autonomous pollination and plant habit, because resource limitation may preclude reproductive features that affect pollinator visitation. In African Gesneriaceae, Hughes *et al.* (2006 and 2007) found that selfing is common among *Streptocarpus* and *Saintpaulia* species. However, *Stenobasipteron wiedemanni*, a Nemestrinidae fly, accounts for most of out-crossing in *S. primulifolius*, showing that even if some species are selfers and have the selfing syndrome (i.e. small flowered species with low herkogamy, Goodwillie *et al.*, 2010), some others depend on pollinators for their reproduction (Potgieter & Edwards, 2005; Hughes *et al.*, 2007).

A possible negative outcome of self-pollination is inbreeding depression. In *Saintapulia ionantha*, high levels of inbreeding depression associated with scarce and small populations (Kolehmainen *et al.* 2009). This result would be one of a few in Gesneriaceae that report inbreeding depression, although not due to pollinator service. There are some populations of Gesneriaceae that are small and with scattered individuals and many are capable of self-fertilize, so is possible that long fruit dispersal, either by animals or by wind, or that long-distance flying pollinators, such as large bees, hummingbirds, bats or large hawkmoths, account for sufficient cross-pollination in those populations. However, more work has to be done to understand the effects of selfing in self-compatible but pollinator-dependent species of Gesneriaceae.

Studies of breeding systems in Gesneriaceae species should evaluate whether self-fertilization is a widespread character or not and possible related mechanisms that affect reproduction. Thus, opening a new frame for investigation in the evolution of breeding systems.

Dichogamy and Herkogamy

Within reproductive strategies temporal and spatial separation of sexual organs has been proposed as a way to avoid sexual interference and to promote cross-fertilization (Lloyd & Webb, 1986; Webb & Lloyd, 1986; Bertin, 1993). Most Gesneriaceae species in our search are dichogamous and among this temporal separation, protandry (staminate phase develops first) is more common than protogyny (i.e. pistillate phase develops before staminate phase). Interestingly, protogynous species described are represented in the Neotropical

Gesneria and *Rhytidophyllum* genera, and in *Oreocharis acaulis,* a Chinese species. *Oreocharis acaulis*, is a bee pollinated species with low pollinator visitation. This species shows protogyny in combination with approach herkogamy and probably promoting cross-pollination by reducing the risk of self-pollination (Guo *et al.*, 2013). Gou *et al.* (2013) also propose that the large duration of stigma receptivity is a reproductive assurance mechanism, because the probability of receiving pollen increases with time.

Gesneria and *Rhytidophyllum* genera are distributed in the Caribbean region and comprise 53 and 19 species, respectively. Among these species, there are protogynous species that are pollinated by hummingbirds (*G. citrina, G. cuneifolia*), by bats and hummingbirds (*G. quisqueyana*) and by bats, hummingbirds, moths and diurnal insects (*G. viridiflora subsp. sintenisii, R. asperum, R. auriculatum, R. grandiflorum* and *R. leucomallon*) (Martén-Rodríguez & Fenster, 2008; Martén-Rodríguez *et al.*, 2009). Furthermore, all experienced low pollinator visitation and only hummingbird-pollinated ones and two species in that study showed autonomous pollination as a reproductive assurance strategy. A particular case of the expression of reproductive assurance is the elongation of stamens after pollination of *Gesneria citrina*, a hummingbird pollinated species (Chen *et al.*, 2009). This a rare example of protogyny in combination with reduced herkogamy. This example represents an extreme case in which the role of pollinator visitation affects floral morphology and reproductive systems.

In self-compatible species, protogyny rather than protandry may be more efficient at reducing sex interference and pollen discounting (Lloyd & Webb, 1986). Wyatt (1983) proposed that protandry is associated with bee pollination, however he did not include vertebrate pollinated systems in his study. Furthermore, Bertin (1993) suggested that protogyny is a better mechanism that assures self-pollination. We expand this suggestion to an ecological view and propose for future works to test for pollen limitation in Gesneriaceae species with protogyny in relation to protandrous congeneric species. Thus, we suggest that pollen limitation rather than pollinator functional group, may be the main factor that promotes protogyny in Gesneriaceae.

Enantiostyly

Enantiostyly is a particular case of herkogamy (i.e. special separation of sexual organs) and is defined as the deflection of the style to the right or left of the main floral axis, but there is pollination success only if a pollinator touches a floral morph with style of the opposite direction (Jesson & Barrett, 2003). This disposition of sexual organs promotes cross fertilization by reducing sex interference (Jesson & Barrett, 2002). In Gesneriaceae, enantiostyly is represented in *Saintpaulia* and *Streptocarpus* genera, in which many species are pollinated by large bees.

Enantiostyly is sometimes associated with buzz pollination by large bees and it promotes out-crossing (Barrett *et al.*, 2000; Buchmann, 1983; de Almeida *et al.*, 2013; Solís-Montero & Vallejo-Marín, 2017). Combination of buzz pollination with enantiostyly is reported for Old World (Cyrtandroideae) species. It is represented in *Saintpaulia teitensis* and *S. confusa*, which are bee pollinated by *Amegilla* spp. (Anthophorini) and apparently bee-pollination may be common in the complete African genus (Kolehmainen & Mutikainen, 2006; Martins, 2008). It is also reported for Chinese *Paraboea rufescens*, which is pollinated by two species of *Bombus* (Apidae: Bombini), *Amegilla* and *Nomia* (Apidae: Anthophorini) (Gao *et al.*, 2006). Enantiostyly with buzz-pollination is a specialized form of out-crossing that apparently has evolved in African and Chinese species of Gesneriaceae, interestingly in hot-spots of diversity (i.e. Taita Hills in Kenya and Yunnan Mountains in China). The fact that special pollination mechanisms are in highly diverse areas, may point to avoidance of competition mechanisms that favour different pollen placement while promoting out-crossing (Barrett, 2002; Jesson & Barrett, 2003), but the association with competition in sympatry is underexplored.

Sex separation at whole plant level

Sex separation within one individual is found when flowers express different sex functions. Sexual separation is proposed to limit pollen interference (Wyatt, 1983). Sex separation may be expressed when some flowers are male, other staminate and others may be hermaphrodite. In the Gesneriaceae, *Besleria triflora* was the only species that presents different functional sexes of flowers. This species is andromonoecious, i.e. some flowers function as males and others are hermaphrodite, and is hummingbird-pollinated. In this species, the presence of male flowers is associated with a slight improvement of reproductive success of the male function, i.e. pollen deposition, but the presence of male flowers increases pollinator-attraction (Podolsky, 1992, 1993). However, to our knowledge there is not any other Gesneriaceae species with andromonoecy or other strategy of sex separation at the whole plant level.

Apomixis

Species of Gesneriaceae in our search did not present apomixis. Apomixis is a kind of asexual reproduction were embryo development is from ovule tissues (Bicknell & Koltunow, 2004). Apomixis can be of two types, either sporophytic or gametophytic, depending on the location of the cells were embryo development initiates. The most common experimental approach to recognize sporophytic apomixis is emasculation and bagging of flowers (Kearns & Inouye, 1993). However, of analysed species, ten reported this treatment. None of the papers analysed, mentioned the presence of apomixis, but more complete descriptions of reproductive systems should be made.

Floral trait evolution

We found 16 papers that evaluated in a phylogenetic context the evolution of floral characters (corolla colour, corolla size, corolla shape, calyx margin, corolla base, symmetry), rewards (nectar volume, nectar sugar concentration), and some evaluated pollination systems among other variables (habit, climatic variables, and/or phenology) (; Harrison *et al.*, 1999; Perret *et al.*, 2003; Hughes *et al.*, 2006; Perret *et al.,* 2007; Martén-Rodríguez *et al.*, 2010; Roalson *et al.*, 2003; Clark *et al.*, 2006, Wang *et al.*, 2010; Clark *et al.*, 2011; Clark *et al.*, 2015; Shulte *et al.*, 2015; Serrano-Serrano *et al.*, 2015; Roalson & Roberts, 2016; Serrano-Serrano *et al.*, 2017). In papers where floral traits were studied under a pollinator selection hypothesis, pollinator systems were either inferred from floral traits (pollination syndrome) or it was actually observed (Perret *et al.*, 2003; Martén-Rodríguez *et al.*, 2010; Clark *et al.*, 2015; Serrano-Serrano *et al.*, 2015; Roalson & Roberts, 2016; Serrano-Serrano *et al.*, 2017; Shulte *et al.*, 2015). Discussions about floral evolution and pollinator mediated selection were approached by assuming pollinator mediated selective pressures based on evidence in other angiosperm species, i.e., following pollination syndrome descriptions. Only three out of 15 papers used field observations of pollinators to determine and guide an adaptive hypothesis through their research programs (Clark *et al.*, 2015; Martén-Rodríguez *et al.*, 2010; Perret *et al.*, 2003).

An interesting result from Serrano-Serrano *et al.* (2017) is that hummingbird pollination was the most represented pollination system and that changes from hummingbird to bee pollination systems accounted for almost twice as the reverse. This article, found that the first change was from bee to hummingbird pollination systems around 20 million years ago (mya), while the reverse was around 12 mya. Furthermore, hummingbirds accounted for most speciation rates in Gesneriaceae. Another interesting outcome from this research, is that of 118 species, 5% of them had pollinator values of predictability higher than 0.9 to 1. This result confirms the utility of pollination syndromes as proxies to identify pollination systems as found by Rosas-Guerrero *et al.* (2014) and Ashworth *et al.* (2015). However, there is still need to carry on field observations, since definitions of pollination syndromes are based on what is known and may be biased by authors own definitions.

Indeed, pollination syndrome has a predictive value when there is scarce knowledge about a species (Ashworth *et al.*, 2015), but in order to test for adaptive hypotheses, more biologically informed data should be taken into account. Interestingly, even if there were incomplete field observations, most papers found an association between pollination systems and particular floral traits, such as corolla colour and shape (e.g. Clark *et al.* 2011; Serrano-Serrano *et al.*, 2015; Shulte *et al.* 2015). However, nectar traits associated with particular pollinators had two trends, nectar volume was associated with particular pollinators but nectar sugar composition was not associated with particular pollinators (Perret *et al.* 2003), suggesting that pollinator mediated selection is not affecting nectar composition in these species. Whether this pattern is shared by other Gesneriaceae species still needs further investigation, since we found only one paper that tested the association between nectar traits and pollination system in Gesneriaceae. However, at a higher taxonomic level of angiosperms, Abrahamczyk *et al.* (2017) found that nectar composition was correlated with specialized pollinator functional groups (bees, bats, flies, hummingbirds, birds, butterflies), but with high variability in within pollinator functional groups, suggesting that pollinators are important selective agents in floral nectar composition but not the only one.

The most supported trend in Gesneriaceae in relation to pollinators was that changes in floral shapes are related to changes in pollinators systems. Both Martén-Rodríguez *et al.*

(2010) and Clark *et al.* (2015) found that floral shape evolution was associated with pollinator systems. The former authors found that wide and open flowers are correlated with bat-pollination systems, while narrower one were correlated with hummingbird pollination. Interestingly, sub campanulate flowers correlate with mixed-pollination systems. The latter authors inferred that laterally compressed corollas are associated with bird-pollination, while campanulate ones are associated with bee-pollination.

Another interesting trend found was related to breeding-systems in *Streptocarpus* species. Hughes *et al.* (2006) found that there are flowers in *Streptocarpus* genus that tend to increase in size coupled with shape and that the plesiomorphic small sizes are possibly associated with self-pollination. Unfortunately, discussion about the association between floral shape and breeding-systems was based on three species with complete documentation of selfing and population genetics, in a 76 species phylogeny, again elaborating an adaptive hypothesis with few data to support it. Nevertheless, their results point to another research area to understand the evolution of floral morphology in Gesneriaceae.

Conclusions

Inconsistencies between some traits and pollinator system (i.e. traits not fitting the classical pollination syndrome definitions) have been interpreted as transitions among syndromes, because some floral traits actually fit other syndromes (e.g. *Paliavana sericiflora* (Gesneriaceae) Sanmartin-Gajardo & Sazima, 2005a; *Abutilon rufinerve*, *A. regnellii*, *A. aff. regnellii* (Malvaceae), (Buzato *et al.*, 1994); *Penstemon roseus* (Scrophulariaceae) Lara & Ornelas, 2008). Furthermore, in the presence of more than one effective pollinator, generalized pollination systems and intermediate floral characters also evolve, for example *Rhytidophyllum* species and *Gesneria pedunculosa* (Martén-Rodríguez *et al.*, 2009). In our search, these were the only species that were reported as mixed-pollinated, suggesting that generalization in pollination systems may be more frequent in islands than in mainland, probably due to more variation in pollination services. Also, there should be a more thorough description of pollination biology in both mainland and island species. As for the presence of secondary pollinators, only Rosas-Guerrero *et al.* (2014) suggested that secondary pollinators are usually the ancestral pollinator group, and in Gesneriaceae species this is probably the case, but more evidence should be gathered. Although, secondary pollinators are less frequent and efficient, their presence may affect selection mediated by primary pollinators and these effects are less known (Thomson, 2003). Although there is a remarkable association between different floral character states (corolla shape, colour and rewards) with particular pollinators, other mechanisms like development or abiotic factors may also promote variation in floral diversity (Wessinger & Hileman, 2016).

Gesneriaceae family represents an interesting system to study different aspects of floral biology and ecology associated with both pollination systems and breeding systems, and within a phylogenetic context.

Literature cited

Anderson, S.H., Kelly, D., Ladley, J.J., Molloy, S. & Terry, J. 2011. Cascading effects of bird functional extinction reduce pollination and plant density. *Science* **331**: 1068–1071.

Ashworth, L., Aguilar, R., Martén-Rodríguez, S., Lopezaraiza-Mikel, M., Avila-Sakar, G., Rosas-Guerrero, V., *et al.* 2015. Pollination syndromes: a global pattern of convergent evolution driven by the most effective pollinator. In: *Evolutionary Biology: Biodiversification from Genotype to Phenotype* (P. Pontarotti, ed.), pp. 203–224. Springer International Publishing, Switzerland.

Ayala, A.V.R. 1986. Aspectos de la relación entre *Thalurania furcata colombica* (Aves Trochilidae) y las flores en que liba, en un bosque subandino. *Caldasia* **XIV**: 68–70.

de Ávila, R. S. 2010. *Besleria longimucronata* y sus ladrones de néctar: ¿cuáles son sus consecuencias sobre el éxito reproductivo en esta supuesta interacción negativa?

Azuma, H., Toyota, M., Asakawa, Y. & Kawano, S. 1996. Naphthalene- a constituent of *Magnolia* flowers. *Phytochemistry* **42**: 999–1004.

Baker, H.G.,Baker, I. & Hodges, S.A. 1998. Sugar composition of nectars and fruits consumed by birds and bats in the Tropics and Subtropics. *Biotropica* **30**: 559–586.

Barrett, S.C.H. 1998. The evolution of mating strategies in flowering plants. *Trends Plant Sci.* **3**: 335–341.

Barrett, S.C.H, Jesson, L.K. & Baker, A. 2000. The evolution of stylar polymorphisms in flowering plants. *Ann. Bot*. **85** (SuppA): 253–265.

Bennett, A.T.D. & Cuthill, I.C. 1994. Ultraviolet vision in birds: what is its function? *Vision Res*. **34**: 1471–1478

Bernardello, L.M., Galetto, L., Jaramillo, J. & Grijalba, E. 1994. Floral nectar chemical composition of some species from Reserva Rio Guajalito, Ecuador. *Biotropica* **26**: 113–116.

Bertin, R.I. 1993. Incidence of monoecy and dichogamy in relation to self-fertilization in angiosperms. *Am. J. Bot.* **80**: 557–560.

Bicknell, R.A. & Koltunow, A.M. 2004. Understanding apomixis: recent advances and remaining conundrums. *Plant Cell* **16**: S228–S245.

Bogacheva-Milkoteva, K., Kozuharova, E. & Claßen-Bockhoff, R. 2013. Pollination ecology of *Haberlea rhodopensis* (Gesneriaceae), a tertiary relict endemic to the Balkan Peninsula. *Phytol. Balc.* **19**: 201–208.

Bogacheva, K., Kozuharova, E. & Kostov, K. 2013. *Haberlea rhodopensis* and its relatives on the Balkans - what do we know about these peculiar Gesneriaceae members so far? *OT Sist. Bot. Derg.* **20**: 119–150.

Boggan, J.K. 1996. Fragrant Gesneriads. *The Gloxinian* **46**.

Boggan, J.K., Skog, L.E. & Roalson, E.H. 2008. A review of the Neotropical genera *Amalophyllon, Niphaea*, and *Phinaea* (Gesneriaceae-Gloxinieae). *Selbyana* **29**: 157–176.

Buchmann, S.L. 1983. Buzz pollination in angiosperms. In: *Handbook of experimental pollination biology* (C. E. Jones & R. J. Little, eds.), pp. 73–113. Van Nostrand Reinhold, New York.

Bush, S.P. & Beach, J.H. 1995. Breeding systems of epiphytes in a tropical montane wet forest. *Selbyana* **16**: 155–158.

Buzato, S., Sazima, M. & Sazima, I. 2000. Hummingbird-pollinated floras at three Atlantic forest sites. *Biotropica* **32**: 824–841.

Buzato, S., Sazima, M. & Sazima, I. 1994. Pollination of three species of *Abutilon* (Malvaceae) intermediate between bat and hummingbird flower syndromes. *Flora* **189**: 327–334.

Camargo, E., Rodrigues, L. da C. & Araújo, A.C. 2011. Pollination biology and reproduction of *Seemannia sylvatica* (Kunth) Hanstein (Gesneriaceae) in the Serra da Bodoquena National Park, Mato Grosso do Sul. *Biota Neotrop.* **11**: 125–130.

Carlson, J.E. & Harms, K.E. 2007. The benefits of bathing buds: Water calyces protect flowers from a microlepidopteran herbivore. *Biol. Lett.* **3**: 405–407.

Castellanos, M.C., Wilson, P. & Thomson, J.D. 2004. ‘Anti-bee’ and ‘pro-bird’ changes during the evolution of hummingbird pollination in *Penstemon* flowers. **17**: 876–885.

Chen, X.-S., Martén-Rodríguez, S., Li, Q.-J. & Fenster, C.B. 2009. Potential autonomous selfing in *Gesneria citrina* (Gesneriaceae), a specialized hummingbird pollinated species with variable expression of herkogamy. *J. Integr. Plant Biol.* **51**: 973–978.

Chittka, L. & Waser, N.M. 1997. Why red flowers are not invisible to bees. *Isr. J. Plant Sci.* **45**: 169–183.

Chittka, L.T. & Thomson, J.D. (eds.). 2004. *Cognitive Ecology of Pollination. Animal Behaviour and floral evolution*, 1st ed. Cambridge University Press, Cambridge, UK.

Clark, J.L., Clavijo, L. & Muchhala, N. 2015. Convergence of anti-bee pollination mechanisms in the Neotropical plant genus *Drymonia* (Gesneriaceae). *Evol. Ecol.* **29**: 355–377.

Clark, J.L., Herendeen, P.S., Skog, L.E. & Zimmer, E.A. 2006. Phylogenetic relationships and generic boundaries in the Episcieae (Gesneriaceae) inferred from nuclear, chloroplast, and morphological data. *Taxon* **55**: 313–336.

Clark, J.R., Ree, R.H., Alfaro, M.E., King, M.G., Wagner, W.L. & Roalson, E.H. 2008. A comparative study in ancestral range reconstruction methods: Retracing the uncertain histories of insular lineages. *Syst. Biol.* **57**: 693–707.

Clark, J.L., Roalson, E.H., Pritchard, R.A., Coleman, C.L., Teoh, V.-H. & Matos, J. 2011. Independent origin of radial floral symmetry in the Gloxinieae (Gesnerioideae: Gesneriaceae) is supported by the rediscovery of *Phinaea pulchella* in Cuba. *Syst. Bot.* **36**: 757–767.

Clark, J.R., Wagner, W.L. & Roalson, E.H. 2009. Patterns of diversification and ancestral range reconstruction in the southeast Asian-Pacific angiosperm lineage *Cyrtandra* (Gesneriaceae). *Mol. Phylogenet. Evol.* **53**: 982–994. Elsevier Inc.

Dalsgaard, B., Kennedy, J.D., Simmons, B.I., Baquero, A.C., Martín González, A.M., Timmermann, A., *et al.* 2018. Trait evolution, resource specialization and vulnerability to plant extinctions among Antillean hummingbirds. *Proc. R. Soc. B Biol. Sci.* **285**: 20172754.

de Almeida, N.M., De Castro, C.C., de Lima Leite, A. V., Novo, R.R. & Machado, I.C. 2013. Enantiostyly in *Chamaecrista ramosa* (Fabaceae-Caesalpinioideae): Floral morphology, pollen transfer dynamics and breeding system. *Plant Biol.* **15**: 369–375.

de Avila, R.S.J. 2010. *Besleria longimucronata* (Gesneriaceae) y sus ladrones de néctar: ¿Cuáles son las consecuencias sobre el éxito reproductivo en esta supuesta interacción negativa? *Ecol. Austral* **20**: 209–213.

Dressler, R.L. 1968a. Observations on orchids and Euglossine bees in Panama and Costa Rica. *Rev. Biol. Trop.* **15**: 143–183.

Dressler, R.L. 1968b. Pollination by Euglossine bees. *Evolution* **22**: 202–210.

Endress, P.K. 1994. *Diversity and evolutionary biology of tropical flowers*.

Faegri, K. 1986. The solanoid flower. *Trans. Bot. Soc. Edinburgh* **45**: 51–59.

Faegri, K. & van der Pijl, L. 1979. *Principles of Pollination Biology*, 3th ed. Pergamon Press, New York.

Feinsinger, P. & Colwell, R.K. 1978. Community organization among Neotropical nectar-feeding birds. *Am. Zool.* **18**: 779–795.

Feinsinger, P., Murray, G.K., Kinsman, S. & Busby, W.H. 1986. Floral neighborhood and pollination success in four hummingbird-pollinated cloud forest. *Ecology* **67**: 449–464.

Fenster, C.B., Armbruster, W.S., Wilson, P., Dudash, M.R. & Thomson, J.D. 2004. Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* **35**: 375–403.

Fenster, C.B. & Martén-Rodríguez, S. 2007. Reproductive assurance and the evolution of pollination specialization. *Int. J. Plant Sci.* **168**: 215–228.

Ferreira, P.A. & Viana, B.F. 2010. Pollination biology of *Paliavana tenuiflora* (Gesneriaceae: Sinningeae) in Northeastern Brazil. *Acta Bot. Brasilica* **24**: 972–977.

Ferrer, M.M. & Good-Avila, S.V. 2007. Macrophylogenetic analyses of the gain and loss of self-incompatibility in the Asteraceae. *New Phytol.* **173**: 401–414.

Fleming, T.H., Geiselman, C. & Kress, W.J. 2009. The evolution of bat pollination: a phylogenetic perspective. *Annals of Botany* **104**:1017–1043.

Fleming, P.A., Hartman, B., Lotz, C.N. & Nicolson, S.W. 2004. Concentration and temperature effects on sugar intake and preferences in a sunbird and a hummingbird. *Func. Ecol.* **18**: 223–232.

Freeman, C.E., Worthington, R.D. & Jackson, M.S. 1991. Floral nectar sugar compositions of some South and Southeast Asian species. *Biotropica* **23**: 568–574.

Galetto, L. & Bernardello, G. 2004. Floral nectaries, nectar production dynamics and chemical composition in six *Ipomoea* species (Convolvulaceae) in relation to pollinators. *Ann. Bot.* **94**: 269–280.

Gao, J.-Y., Ren, P.-Y., Yang, Z.-H. & Li, Q.-J. 2006. The pollination ecology of *Paraboea rufescens* (Gesneriaceae): a buzz-pollinated tropical herb with mirror-image flowers. *Ann. Bot.* **97**: 371–376.

Gardener, M.C. & Daehler, C.C. 2006. Documenting floral visitors to rare Hawaiian plants using automated video recordings. *Pacific Conserv. Biol.* **12**: 189–194.

Goodwillie, C., Sargent, R.D., Eckert, C.G., Elle, E., Geber, M.A., Mark, O., et al. 2010. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytol.* **185**: 311–321.

Grant, V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proc. Natl. Acad. Sci. USA* **91**: 3–10.

Guo, Y.-F., Wang, Y.-Q. & Weber, A. 2013. Floral ecology of *Oreocharis acaulis* (Gesneriaceae): an exceptional case of "preanthetic" protogyny combined with approach herkogamy. *Flora* **208**: 58–67.

Guo, Y.F. & Wang, Y.Q. 2014. Floral ecology of *Oreocharis pumila* (Gesneriaceae): A novel case of sigmoid corolla. *Nord. J. Bot.* **32**: 215–221.

Harrison, C.J., Möller, M. & Cronk, Q.C.B. 1999. Evolution and development of floral diversity in *Streptocarpus* and *Saintpaulia*. *Ann. Bot.* **84**: 49–60.

Heithaus, E. R., Fleming, T. H., & Opler, P. A. 1975. Foraging patterns and resource utilization in seven species of bats in a seasonal tropical forest. *Ecology* **56**: 841–854.

Hughes, M., MacMaster, G., Möller, M., Bellstedt, D.U. & Edwards, T.J. 2006. Breeding system of a plesiomorphic floral type: an investigation of small flowered *Streptocarpus* (Gesneriaceae) species. *Plant Syst. Evol.* **262**: 13–24.

Hughes, M., Möller, M., Edwards, T.J., Bellstedt, D.U. & de Villiers, M.J. 2007. The impact of pollination syndrome and habitat on gene flow: a comparative study of two *Streptocarpus* (Gesneriaceae) species. *Am. J. Bot.* **94**: 1688–1695.

Jesson, L.K. & Barrett, S.C.H. 2002. Solving the puzzle of mirror-image flowers. *Nature* **417**: 707.

Jesson, L.K. & Barrett, S.C.H. 2003. The comparative biology of mirror-image flowers. *Int.*

J. Plant Sci. **164**: 237–249.

Johnson, S.D. & Nicolson, S.W. 2008. Evolutionary associations between nectar properties and specificity in bird pollination systems. *Biol. Lett.* **4**: 49–52.

Johnson, M.A., Price, D.K., Price, J.P. & Stacy, E.A. 2015. Postzygotic barriers isolate sympatric species of *Cyrtandra* (Gesneriaceae) in Hawaiian montane forest understories. *Am. J. Bot.* **102**: 1870–1882.

Johnson, M.A., Pillon, Y., Sakishima, T., Price, D.K. & Stacy, E.A. 2019. Multiple colonizations, hybridization and uneven diversification in *Cyrtandra* (Gesneriaceae) lineages on Hawai'i Island. *J. Biogeogr.* 46: 1178–1196.

Jones, E.C. & Buchmann, S.L. 1974. Ultraviolet floral patterns as functional orientation cues in hymenopterous pollination systems. *Anim. Behav.* **22**: 481–485.

Jones, E.J. & Vickers, P.R. 1972. Ornithophily and extrafloral color patterns in *Columnea florida* Morton (Gesneriaceae). *Bull. South. Acad. Sci.* **71**: 113–116.

Kato, M., Ichino, T., Hotta, M., Abbas, I. & Okada, H. 1989. Flower visitors of 32 plant spcies in West Sumatra. *Ocassional Pap.* **16**: 15–32.

Kato, M., Itino, T. & Nagamitsu, T. 1993. Melittophily and ornithophily of long-tubed flowers in Zingiberaceae and Gesneriaceae in West Sumatra. *Tropics* **2**: 129–142.

Kay, K.M., Voelckel, C., Yang, J.Y., Hufford, K.M., Kaska, D.D. & Hodges, S.A. 2006. Floral characters and species diversification. In: *Ecology and Evolution of Flowers* (L. D. Harder & S. C. H. Barrett, eds.), pp. 311–325. Oxford University Press, UK.

Kearns, C.A. & Inouye, D.W. 1993. *Techniques for Pollination Biologists*, 1st ed. University Press of Colorado, Boulder, Colorado, US.

Kolehmainen, J.K. & Mutikainen, P. 2006. Reproductive ecology of three endangered African violet (*Saintpaulia* H. Wendl.) species in the East Usambara Mountains, Kenya. *Afr. J. Ecol.* **44**: 219–227.

Kolehmainen, J., Korpelainen, H. & Mutikainen, P. 2009. Inbreeding and inbreeding depression in a threatened endemic plant, the African violet (*Saintpaulia ionantha* ssp. *grotei*), of the East Usambara Mountains, Tanzania. *Afr. J. Ecol.* **48**: 576–587.

Lara, C. & Ornelas, J.F. 2008. Pollination ecology of *Penstemon roseus* (Plantaginaceae), an endemic perennial shifted toward hummingbird specialization? *Plan Syst. Evol.* **271**: 223–237.

Ling, S.-J., Meng, Q.-W., Tang, L. & Ren, M.-X. 2017. Pollination syndromes of Chinese Gesneriaceae: a comparative study between Hainan Island and neighboring regions. *Bot. Rev.* **83**: 59–73.

Lloyd, D.G. & Webb, C.J. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy of pollen and stigmas in angiosperms. *New Zeal. J. Bot.* **24**: 135–162.

Martel, C., Gerlach, G. & Ayasse, M. 2019. Pollination ecology of the Neotropical gesneriad *Gloxinia perennis*: chemical composition and temporal fluctuation of floral perfume. *Plant Biology* **21**: 723–731.

Martén-Rodríguez, S., Almarales-Castro, A. & Fenster, C.B. 2009. Evaluation of pollination syndromes in Antillean Gesneriaceae: evidence for bat, hummingbird and generalized flowers. *J. Ecol.* **97**: 348–359.

Martén-Rodríguez, S. & Fenster, C.B. 2008. Pollination ecology and breeding systems of five *Gesneria* species from Puerto Rico. *Ann. Bot.* **102**: 23–30.

Martén-Rodríguez, S., Fenster, C.B., Agnarsson, I., Skog, L.E. & Zimmer, E.A. 2010. Evolutionary breakdown of pollination specialization in a Caribbean plant radiation. *New Phytol.* **188**: 403–417.

Martén-Rodríguez, S., Quesada, M., Castro, A.A., Lopezaraiza-Mikel, M. & Fenster, C.B. 2015. A comparison of reproductive strategies between island and mainland Caribbean Gesneriaceae. *J. Ecol.* **103**: 1190–1204.

Martins, D.J. 2008. Pollination observations of the African violet in Taita Hills, Kenya. *J. East African Nat. Hist.* **97**: 33–42.

Medel, R., Botto-Mahan, C. & Kalin-Arroyo, M. 2003. Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. *Ecology* **84**: 1721–1732.

Melendez-Ackerman, E.J. & Campbell, D.R. 1998. Adaptive significance of flower color and inter-trait correlations in an *Ipomopsis* hybrid zone. *Evolution* **52**: 1293–1303.

Michener, C.D. 2007. *The bees of the world*, 2nd ed. (953, ed). Johns Hopkins University Press, Baltimore, Maryland.

Mitchell, R.J. 1993. Adaptive significance of *Ipomopsis aggregata* nectar production: observation and experiment in the field. *Evolution* **47**: 25–25.

Möller, M. & Clark, J.L. 2013. The state of molecular studies in the family Gesneriaceae: a review. *Selbyana* **31**: 95–125.

Morley, B.D.F. 1971. A hybrid swarm between two hummingbird-pollinated species of *Columnea* (Gesneriaceae) in Jamaica. *Bot. J. Linn. Soc.* **64**: 81–96.

Ohashi, K. & Thomson, J.D. 2009. Trapline foraging by pollinators: Its ontogeny, economics and possible consequences for plants. *Ann. Bot.* **103**: 1365–1378.

Ornelas, J.F. & Ordano, M. 2007. Nectar removal effects on seed production in *Moussonia deppeana* (Gesneriaceae), a hummingbird-pollinated shrub. *Ecoscience* **14**: 117–123.

Perret, M., Chautems, A., Spichiger, R., Barraclough, T.G. & Savolainen, V. 2007. The geographical pattern of speciation and floral diversification in the neotropics: the tribe

Sinningieae (Gesneriaceae) as a case study. *Evolution* **61**: 1641–1660.

Perret, M., Chautems, A., Spichiger, R., Kite, G. & Savolainen, V. 2003. Systematics and evolution of tribe Sinningeae (Gesneriaceae): evidence from phylogenetic analyses of six plastid DNA regions and nuclear ncpGS. *Am. J. Bot.* **90**: 445–460.

Perret, M., Chautems, A., Spichiger, R., Peixoto, M. & Savolainen, V. 2001. Nectar sugar composition in relation to pollination syndromes in Sinningieae (Gesneriaceae). *Ann. Bot.* **87**: 267–273.

Podolsky, R.D. 1992. Strange floral attractors: pollinator attraction and the evolution of plant sexual systems. *Science* **258**: 791–793.

Podolsky, R.D. 1993. Evolution of a flower dimorphism - how effective is pollen dispersal by “male” flowers? *Ecology* **74**: 2255–2260.

Potgieter, C.J. & Edwards, T.J. 2005. The *Stenobasipteron wiedemanni* (Diptera, Nemestrinidae) pollination guild in Eastern Southern Africa. *Ann. Missouri Bot. Gard.* **92**: 254–267.

Pregitzer, K.S. 2003. Woody plants, carbon allocation and fine roots. *New Phytol.* 158: 421–424.

Pu, G.-Z., Pan, Y.-M., Tang, S.-C., Wei, C.-Q. & Chen, Q.-X. 2009. Pollination biology and reproductive allocation of *Chirita gueilinensis* (Gesneriaceae). *Bull. Bot. Res.* **29**: 169–175.

Raduski, A.R., Haney, E.B. & Igic, B. 2011. The expression of self-incompatibility in angiosperms in bimodal. *Evolution* **66**: 1275–1283.

Raguso, R.A. 2008. Wake up and smell the roses: the ecology and evolution of floral scent. *Annu. Rev. Ecol. Evol. Syst.* **39**: 549–569.

Rahman, W. 2011. Flower biology of four epiphytic Malesian gesneriads. *Gard. Bull.*

Singapore **63**: 485–493.

Ramírez-Aguirre, E., Martén-Rodríguez, S. & Ornelas, J.F. 2016. Floral variation, nectar production, and reproductive success of two *Drymonia* (Gesneriaceae) species with mixed pollination syndromes. *Int. J. Plant Sci.* **177**: 469–480.

Ramírez-Aguirre, E., Martén-Rodríguez, S., Quesada-Avila, G., Quesada, M., Martínez-Díaz, Y., Oyama, K., *et al.* 2019. Reproductive isolation among three sympatric *Achimenes* species: pre- and post-pollination components. *Am. J. Bot.* **106**:1021–1031.

Riveros, M., Humaña, A.M. & Arroyo, M.K. 1996. Sistemas de reproducción en especies del bosque valdiviano. *Int. J. Exp. Bot.* **58**: 167–176.

Roalson, E.H. & Roberts, W.R. 2016. Distinct processes drive diversification in different clades of Gesneriaceae. *Syst. Biol.* **65**: 662–684.

Roalson, E.H., Skog, L.E. & Zimmer, E.A. 2003. Phylogenetic relationships and the diversification of floral form in *Achimenes* (Gesneriaceae). *Syst. Bot.* **28**: 593–608.

Rodrigues, L. & Rodrigues, M. 2014. Flowers visited by hummingbirds in the open habitats of the southeastern Brazilian mountaintops: species composition and seasonality. *Brazilian J. Biol.* **74**: 659–676.

Roelofs, F.M. 1979. The reproductive biology of *Cyrtandra grandiflora* (Gesneriaceae) on Oahu. *Pacific Sci.* **33**: 223–231.

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J.M., *et al.* 2014. A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett.*, doi: 10.1111/ele.12224.

Rosas-Guerrero, V., Quesada, M., Armbruster, W.S., Pérez-Barrales, R. & Smith, S.D.W. 2011. Influence of pollination specialization and breeding system on floral integration and phenotypic variation in *Ipomoe*a. *Evolution* **65**: 350–364.

Sanmartin-Gajardo, I. & Freitas, L. 1999. Hummingbird pollination in *Besleria longimucronata* Hoene (Gesneriaceae) in Southeastern Brazil. *Biociencias* **7**: 13–24.

SanMartin-Gajardo, I. & Sazima, M. 2004. Non-euglossine bees also function as pollinators of *Sinningia* species (Gesneriaceae) in southeastern Brazil. *Plant Biol.* **6**: 506–512.

Sanmartin-Gajardo, I. & Sazima, M. 2005a. Chiropterophily in Sinningieae (Gesneriaceae): *Sinningia brasiliensis* and *Paliavana prasinata* are bat-pollinated, but *P. sericiflora* is not. Not yet? *Ann. Bot.* **95**: 1097–1103.

Sanmartin-Gajardo, I. & Sazima, M. 2005b. Espécies de *Vanhouttea* Lem. e *Sinningia* Nees (Gesneriaceae) polinizadas por beija-flores: interações relacionadas ao hábitat da planta e ao néctar. *Rev. Bras. Botânica* **28**: 441–450.

Sanmartin-Gajardo, I. & Vianna, J.R.S. 2010. Pollination of *Nematanthus brasiliensis*: an epiphytic Gesneriaceae endemic to the Southeastern Atlantic forests of Brazil. *Selbyana* **30**: 212–216.

Sazima, M., Buzato, S., & Sazima, I. 1999. Bat-pollinated flower assemblages and bat visitors at two Atlantic forest sites in Brazil. *Annals of Botany* **83**: 705–712.

Schemske, D.W. & Bradshaw, H.D. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl. Acad. Sci.* **96**: 11910–11915.

Schiestl, F.P. 2015. Ecology and evolution of floral volatile-mediated information transfer in plants. *New Phytol.* **206**: 571–577.

Schiestl, F.P., Ayasse, M., Taylor, R.P. & Micolich, A.P. 1999. Orchid pollination by sexual swindle. *Nature* 421–422.

Schiestl, F.P. & Johnson, S.D. 2013. Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* **28**: 307–315.

Serrano-Serrano, M.L., Perret, M., Guignard, M., Chautems, A., Silvestro, D. & Salamin, N. 2015. Decoupled evolution of floral traits and climatic preferences in a clade of Neotropical Gesneriaceae. *BMC Evolutionary Biology* **15**: 1–12.

Serrano-Serrano, M.L., Rolland, J., Clark, J.L., Salamin, N. & Perret, M. 2017. Hummingbird pollination and the diversification of angiosperms: an old and successful association in Gesneriaceae. *Proc. R. Soc. B Biol. Sci.* **284**.

Shulte, L.J., Clark, J.L., Novak, S.J., Jeffries, S.K. & Smith, J.F. 2015. Speciation within *Columnea* Section Angustiflora (Gesneriaceae): islands, pollinators and climate. *Mol. Phylogenet. Evol.* **84**: 125–144.

Simpson, B.B. & Neff, J.L. 1981. Floral rewards: alternatives to pollen and nectar. *Ann. Missouri Bot. Gard.* **68**: 301–322.

Smith, S.D.W. 2010. Using phylogenetics to detect pollinator-mediated floral evolution. *New Phytol.* **188**: 354–363.

Smith-Ramírez, C., Martínez, P., Núñez, M., González, C. & Armesto, J.J. 2005. Diversity, floral visitation frequency and generalism of pollinators in temperate rainforests of Chiloé Island, Chile. *Bot. J. Linn. Soc.* **147**: 399–416.

Snow, B.K. 1977. Feeding behavior of two hummingbirds in a Costa Rican montane forest. *Wilson Bull.* **89**: 613–616.

Snow, D.W. & Texeira, D.L. 1982. Hummingbirds and their flowers in the coastal mountains of southeastern Brazil. *J. Ornithol.* **123**: 446–450.

Solís-Montero, L. & Vallejo-Marín, M. 2017. Does the morphological fit between flowers and pollinators affect pollen deposition? An experimental test in a pollinated species with anther dimorphism. *Ecol. Evol.* **7**: 2706–2715.

Stebbins, G.L. 1970. Adaptive radiation of reproductive characteristics in Angiosperms, I:

Pollination mechanisms. *Annu. Rev. Ecol. Syst.* **1**: 307–326.

Stein, K. & Hensen, I. 2013. The reproductive biology of two understory plants in the Atlantic rain forest, Brazil. *Ecol. Res.* **28**: 593–602.

Steiner, K.E. 1985. The role of nectar and oil in the pollination of *Drymonia serrulata* (Gesneriaceae) by *Epicharis* bees (Anthophoridae) in Panama. *Biotropica* **17**: 217.

Stiles, F.G. & Freeman, C.E. 1993. Patterns in floral nectar characteristics of some bird-visited plant species from Costa Rica. *Biotropica* **25**: 191–205.

Strauss, S.Y. & Whittall, J.B. 2006. Non-pollinator agents of selection. In: *Ecology and Evolution of Flowers* (L. D. Harder & S. C. H. Barrett, eds), pp. 120–135. Oxford University Press, New York.

Tang, S.-C., Pu, G.-Z., Pan, Y.-M., Zou, R. & Wei, C.-Q. 2009. Pollination biology of *Chirita lutea* Yan Liu et Y. G. Wei in China (Gesneriaceae). *J. Trop. Subtrop. Bot.* **17**: 328–333.

Thomson, J. 2003. When is it Mutualism? (American Society of Naturalists Presidential Address, 2001). *Am. Nat.* **162**: S1–S9.

Tiedge, K. & Lohaus, G. 2017. Nectar sugars and amino acids in day- and night-flowering *Nicotiana* species are more strongly shaped by pollinators' preferences than organic acids and inorganic ions. *PLoS One* **12**: 1–25.

Utech, F.H. & Kawano, S. 1975. Spectral polymorphisms in angiosperm flowers determined by differential ultraviolet reflectance. *Bot. Mag. Tokyo* **88**: 9–30.

Vogel, S. 1966. Parfümsammelnde bienen als Bestäuber von Orchidacen und Gloxinia. *Österr Voit. Zeit* **113**: 302–361.

Vokou, D., Petanidou, T. & Bellos, D. 1990. Pollination ecology and reproductive potential of *Jankaea heldreichii* (Gesneriaceae); a tertiary relict on Mt Olympus, Greece. *Biol.*

Conserv. **52**: 125–133.

Wang, Y.-B., Liang, H.-W., Mo, N.-B., Qin, K.-P. & Tang, G.-G. 2011. Flower phenology and breeding system of rare and endangered *Dayaoshania cotinifolia*. *Acta Bot. Borealis-Occidentalia Sin.* **31**: 958–965.

Wang, Y.-Z., Liang, R.-H., Wang, B.-H., Li, J.-M., Qiu, Z.-J., Li, Z.-Y., *et al.* 2010. Origin and phylogenetic relationships of the Old World Gesneriaceae with actinomorphic flowers inferred from ITS and trnL-trnF sequences. *Taxon* **59**: 1044–1052.

Wang, C.-N., Möller, M. & Cronk, Q.C.B. 2004. Aspects of sexual failure in the reproductive process of a rare bulbiliferous plant, *Titanotrichum oldhamii* (Gesneriaceae), in subtropical Asia. *Sex. Plant Reprod.* **17**: 23–31.

Webb, C.J. & Lloyd, D.G. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. herkogamy. *New Zeal. J. Bot.* **24**: 163–178.

Weber, A. 2004. Gesneriaceae. In: *Flowering plants Dycotiledons*, pp. 63–158. Springer-Verlag, Berlin.

Wen, F., Fu, L.-F. & Wei, Y.-G. 2012. Pollination biology of *Primulina repanda var. guilinensis* and *P. glandulosa var. yangshuoensis*. *Guihaia* **32**: 571–578.

Wessinger, C.A., Hileman, L. & Rausher M.D. 2014. Identification of major quantitative trait loci underlying floral pollination syndrome divergence in Penstemon. *Phil. Trans. R. Soc. B*. **369**: 20130349.

Whitehead, M.R., Lanfear, R., Mitchell, R.J. & Karron, J.D. 2018. Plant mating systems often vary widely among populations. *Front. Ecol. Evol.* **6**: 1–9.

Wiehler, H. 1976. A report on the classification of *Achimenes, Eucodonia, Gloxinia, Goyazia*, and *Anetanthus* (Gesneriaceae). *Selbyana* **1**: 374–401.

Wiehler, H. 1977. New genera and species of Gesneriaceae from the Tropics. *Selbyana* **2**:

67–132.

Wolowski, M., Farias, C.S., Ashman, T.-L. & Freitas, L. 2012. Predominance of self-compatibility in hummingbird-pollinated plants in the Neotropics. *Naturwissenschaften*, doi: 10.1007/s00114-012-0995-0.

Wyatt, R. 1983. Plant pollinator interactions and the evolution of breeding systems. In: *Pollination Biology* (L. Real, ed), pp. 51–95. Academic Press, Orlando, Florida.

Zimmer, E.A., Roalson, E.H., Skog, L.E., Boggan, J.K. & Idnurm, A. 2002. Phylogenetic relationships in the Gesnerioideae (Gesneriaceae) based on nrDNA and cpDNA trnL-F and trnE-T spacer region sequences. *Am. J. Bot.* **89**: 296–311.

Tables

Table 1. Primary and secondary pollinators are described for different pollination systems in Gesneriaceae species. Primary pollinators are in the top line with secondary pollinator groups below in parenthesis.

Bat (5)	**Bee (42)**	**Hummingbird (77)**	**Butterfly (5)**
Hummingbirds (2)	Butterfly (2)	Butterfly (1)	Wasp (1)
	Moth/hawkmoth (1)	Other hummingbirds (7)	
	Hummingbird (1)		
	Other bees (small bees or euglossine bees) (4)		
	Other insects (3)		

Table 2. Mean nectar volume (±SD) produced by Gesneriaceae species according to their main functional group of pollinators. N is the number of species with reported nectar values.

Pollination system	**Mean (ul)**	**Range (ul)**	**N**
Bat	83.5 ± 2.12	82-85	2
Bee (different families) **Euglossine bee**	19.3 ± 42.42 2.4	0.4 -158.6	19 1
Hawkmoth + Moth	9.0		1
Butterfly	3.0 ± 2.12	1.5-6.6	5
Hummingbird	15.9 ± 15.09	0-87	44

Table 3. Floral rewards of Gesneriaceae species by pollinator functional group. Numbers in parentheses are the number of species with reported rewards.

Pollination system	Reward
Bat	nectar (4)
Bee	nectar, oil, pollen (26)
Bee-Euglossine	fragrance (1), nectar (4)
Hummingbird + Birds	nectar (65/3)
Lepidoptera	nectar (7)

Table 4. Mean (±SD) reported or calculated self-compatibility index (SCI, hand self-pollination/ hand cross-pollination) and auto-fertility index (AFI; autonomous/hand cross-pollination) of Gesneriaceae species according to different main pollinator functional group. Autonomous and hand-cross pollination treatments were averaged among species by pollinator group when present. Numbers in parenthesis are the numbers of species with data of reproductive systems.

Pollination system	SCI	AFI
Bat		1.0 (1)
Bee	1.07 ± 0.76 (5)	0.23 ± 0.32 (11)
Bee-Euglossini		0.38 ± 0.36 (3)
Butterfly		0.99 (1)
Hawkmoth/Moth	1.44 (1)	1 (assumed pollinator)
Hummingbird	0.88 ± 0.22 (19)	0.26 ± 0.34 (37)
Mixed	0.88 ± 0.07 (3)	0.09 ± 0.08 (12)
Specialized	0.94 ± 0.39 (25)	0.28 ± 0.35 (54)

Figures

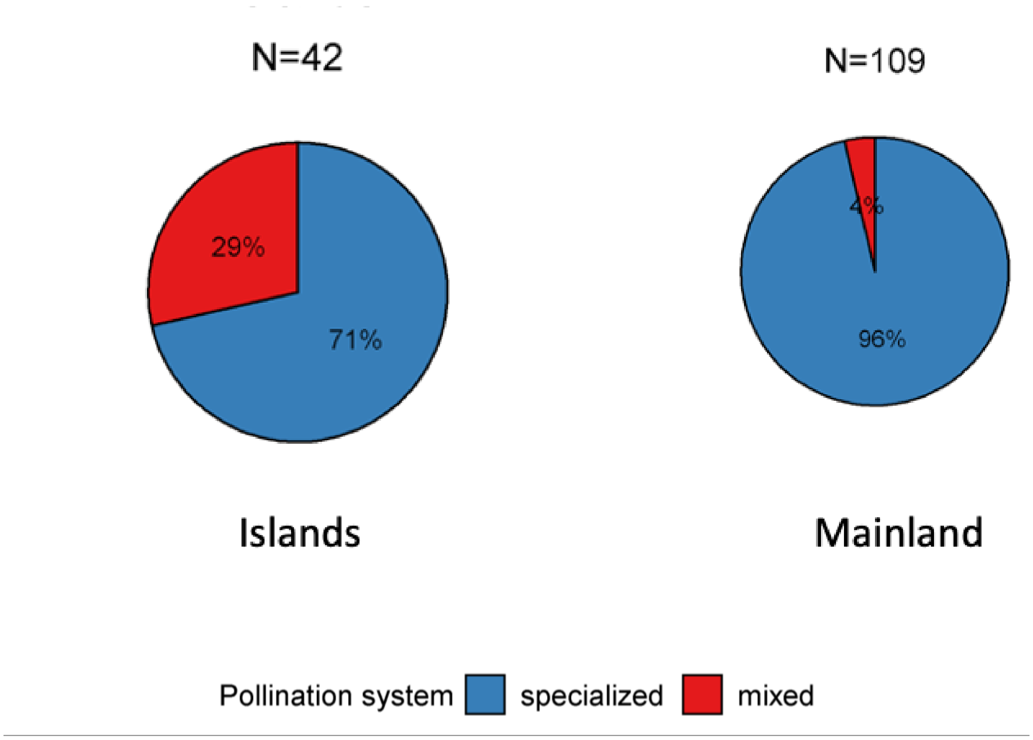


Figure 1. Specialized and mixed pollination systems represented in island and mainland.

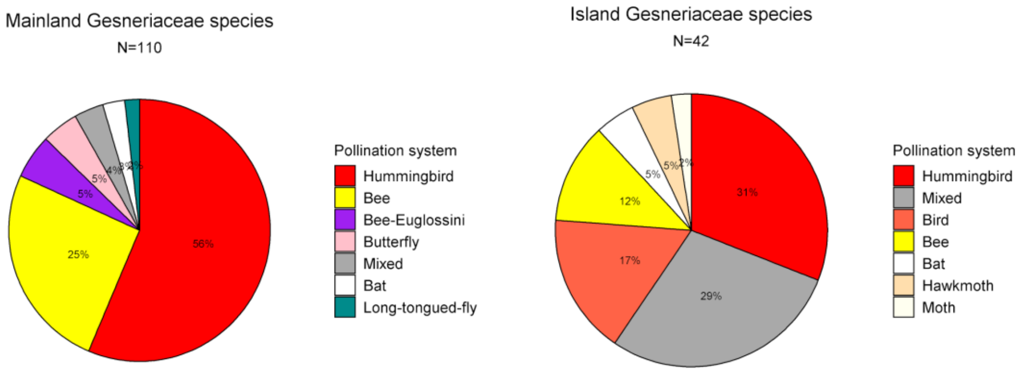


Figure 2. Documented pollinators represented in mainland and island habitats.

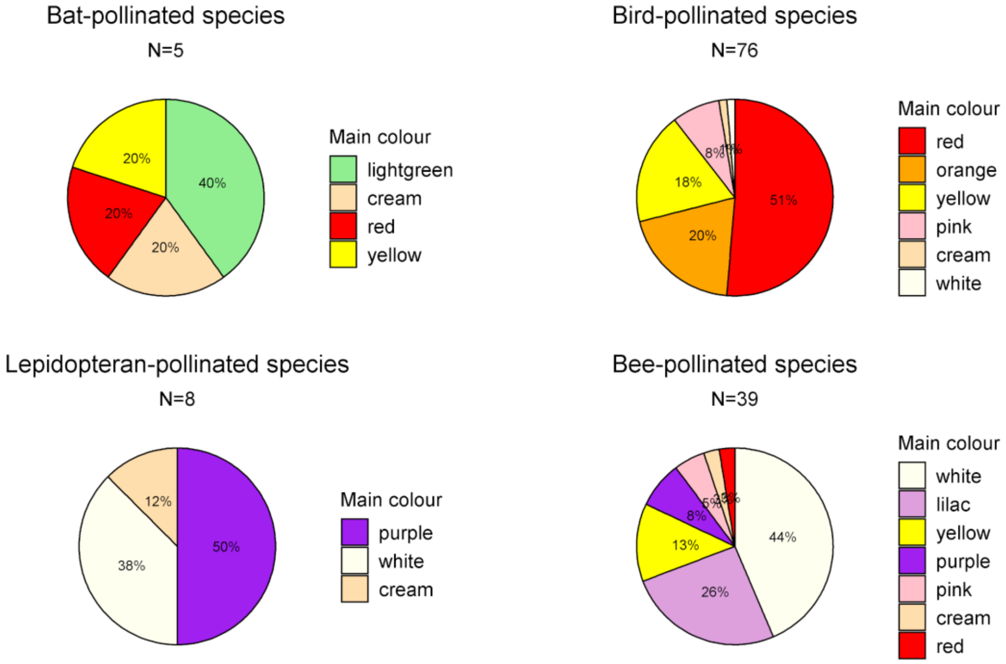


Figure 3. Corolla colours diversity in 128 Gesneriaceae species ordered by pollination system.

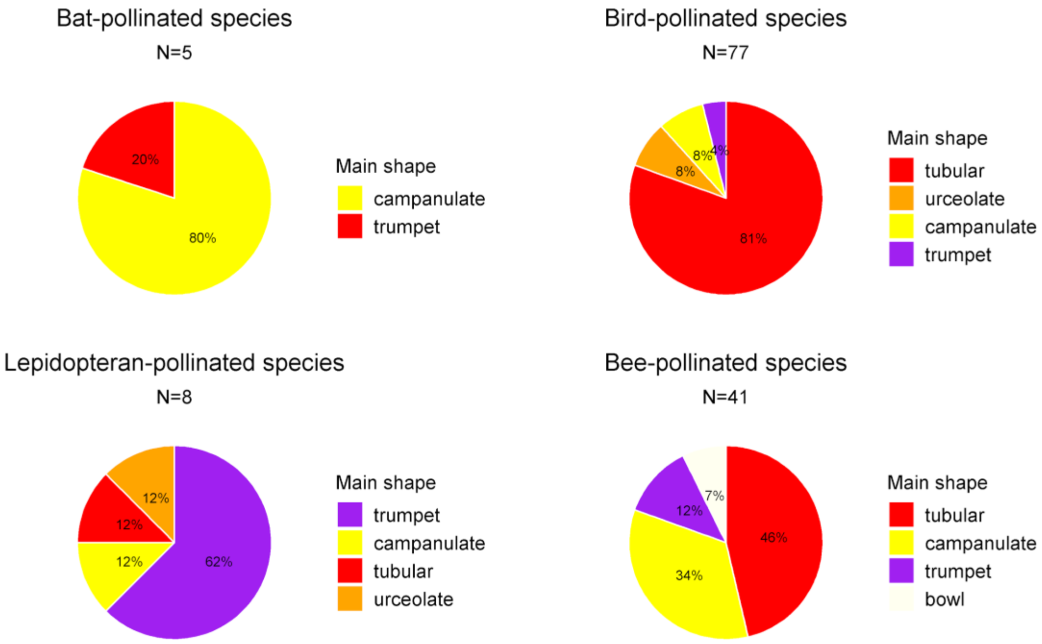


Figure 4. Corolla shapes in 131 Gesneriaceae species ordered by pollination systems.

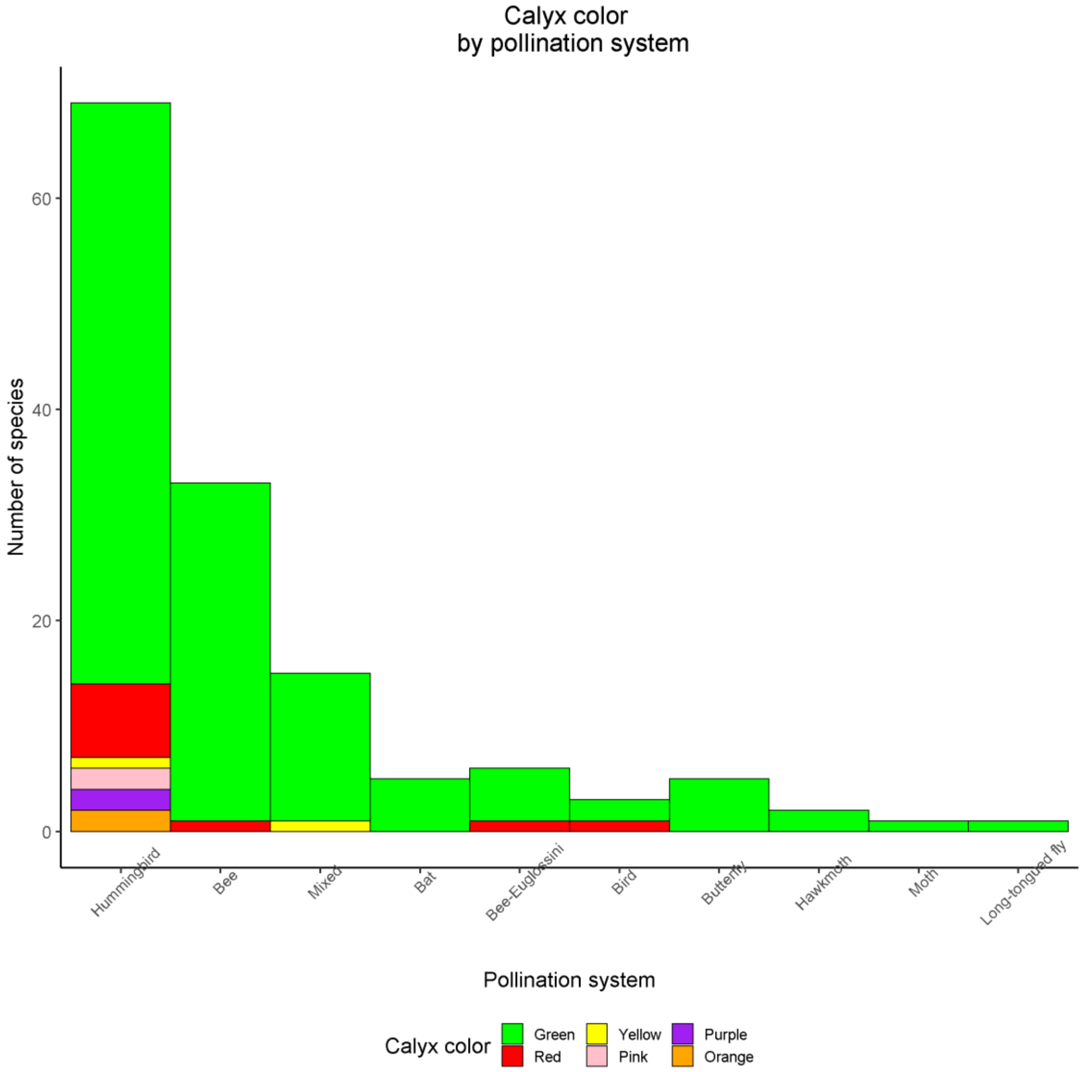
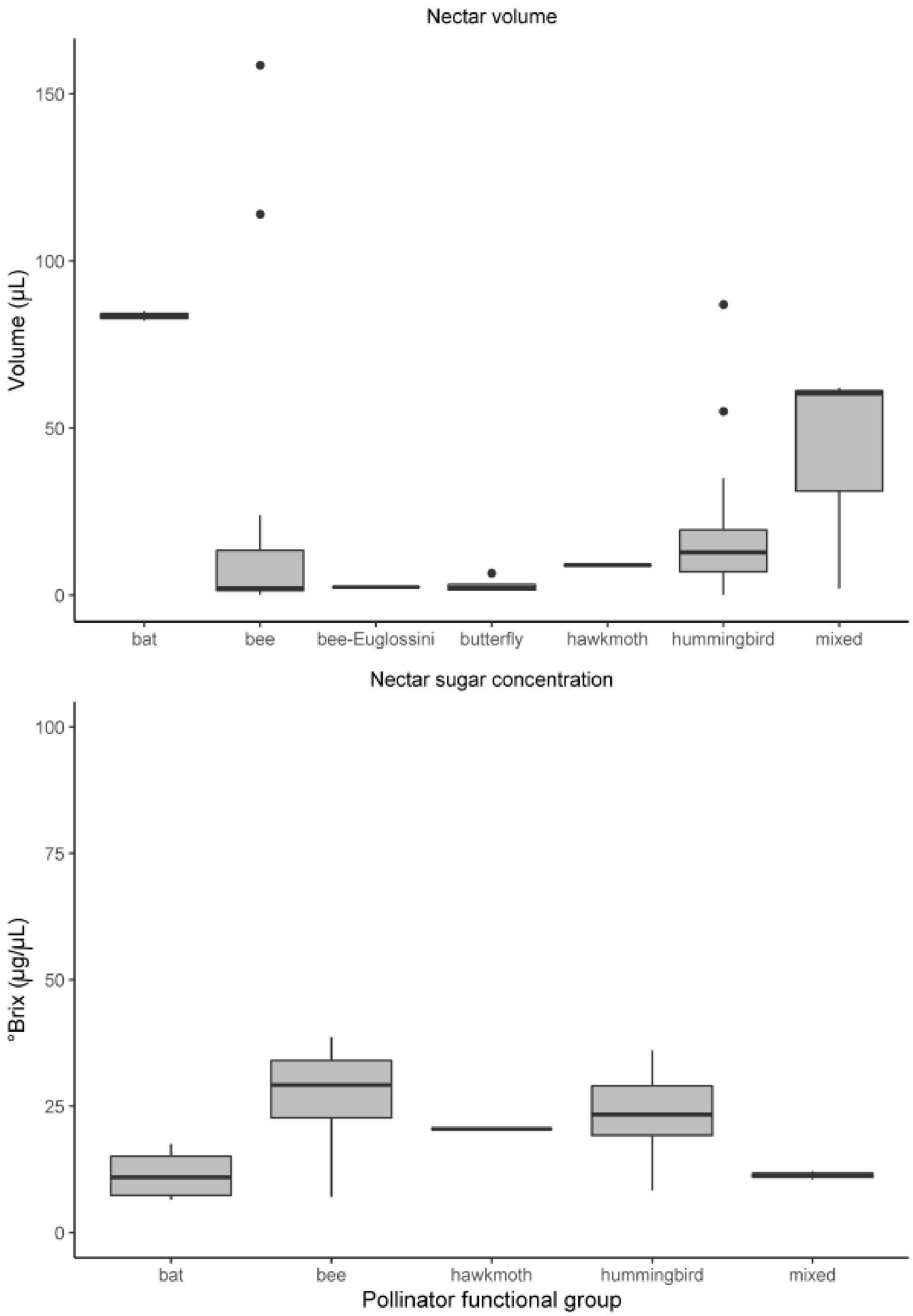


Figure 5. Calyx colors in 145 Gesneriaceae species from across the world.


Nectar volume

Nectar sugar concentration

Figure 6. Nectar traits in 75 Gesneriaceae species from across the world.

DISCUSIÓN Y CONCLUSIÓN GENERAL

Los resultados derivados de esta tesis sugieren que los polinizadores son importantes tanto para la diversificación floral como para el mantenimiento de la identidad genética de las especies de *Achimenes*. El efecto de las presiones selectivas ejercidas por los polinizadores se observa en la presencia de síndromes de polinización en los que existe una clara delimitación de la morfología floral. Además, los síndromes de polinización, entendidos como combinaciones de caracteres florales que atraen y recompensan polinizadores, se repiten en diferentes puntos de la filogenia del grupo *Achimenes*, independientemente de la cercanía evolutiva que compartan las especies. Dicho patrón de surgimiento independiente de los síndromes de polinización sugiere la convergencia adaptativa debida a presiones selectivas similares de los polinizadores.

Sin embargo, la importancia de los polinizadores no es exclusiva del género *Achimenes*. En la familia Gesneriaceae se ha demostrado en diferentes clados la selección mediada por los polinizadores que permite cambios en el volumen de néctar, el color de la corola y su forma, aunque no en el contenido de azúcar. No sólo es en la familia Gesneriaceae de relativamente joven aparición que los polinizadores ejercen presiones selectivas importantes. En los géneros *Penstemon, Ruellia*, *Costus*, *Antirrhinum, Gladiolus*, *Lobelia*, *Hakea, Iochroma, Aquilegia*, *Mimulus*, *Lapeirousia*, *Aphelandra*, *Passiflora* entre otros, los polinizadores consistentemente promueven patrones evolutivos en los que suelen coincidir los cambios de polinizador con cambios en el color, largo de la corola, forma de la corola, simetría de la corola, la protrusión de las anteras y estigmas; en las recompensas como el contenido de azúcar, el volumen del néctar, o la presencia de mecanismos de polinización más activos en los que el polinizador al entrar en contacto con la flor favorece la entrega de polen (Abrahamczyk *et al.*, 2014; Valente *et al.*, 2012; Tripp & Manos, 2010; Goldblatt *et al.*, 1995; Whittall & Hodges, 2007; Kay *et al.*, 2005; Hanley *et al.*, 2009; Givnish *et al.*, 2009; Smith *et al.*, 2008; Beardsley *et al.*, 2003; McDade, 1992; Wilson *et al.*, 2007).

En los ejemplos citados anteriormente, se observa que los diferentes polinizadores promueven cambios simultáneos entre diferentes caracteres florales, apoyando la idea de que la morfología floral y las recompensas están más integrados entre sí que con otras partes vegetales, idea propuesta inicialmente por Berg (1960). Diferentes trabajos han encontrado que la estrecha relación entre partes florales está frecuentemente asociada a la función reproductiva de transferencia de polen y a la atracción de polinizadores (e.g.Esteve-Altava 2017; Armbruster & Wege, 2019). Dicha relación funcional entre partes florales es resultado de cambios genéticos y del desarrollo, algunos codificados por loci de gran efecto y otros regulados por factores de transcripción con efectos pleiotrópicos heredables (Yuan *et al.*, 2013; Wessinger *et al.*, 2014; Wózniak & Sicard 2018). Se ha propuesto que los cambios en los fenotipos complejos, como las flores, pueden ser a través de patrones de desarrollo que sean más fáciles de cambiar, y que afectan los patrones evolutivos, ya sea promoviendo o limitando ciertos tipos de cambios en los caracteres fenotípicos (Felice *et al.*, 2019).

Las flores son fenotipos complejos que responden a la selección mediada de los polinizadores, y la integración y la modularidad entre sus partes, da forma al concepto de síndromes de polinización. La integración fenotípica implica la covariación entre partes de los organismos derivada de interacciones funcionales, genéticas y del desarrollo; mientras que la modularidad es la mayor integración entre ciertas partes de los organismos, funcionando como una unidad cohesiva y flexible y separada de otras unidades (Armbruster *et al.*, 2014; Klingenberg *et al.*, 2001). Entonces, los síndromes de polinización representan una hipótesis adaptativa y funcional en la que la mayor eficiencia en la polinización favorece ciertas correlaciones.

Este trabajo refleja la importancia de los estudios comparativos, en los que los caracteres florales de miembros de clados de angiospermas son analizados dentro de un contexto filogenético que permite evaluar hipótesis adaptativas.

Abrahamczyk, S. et al. 2014. Escape from extreme specialization: passion flowers, bats and the sword-billed hummingbird. *Proc. of the Royal Society B* **281**

Aigner, P.A. 2001. Optimality modeling and fitness trade-offs: when should plant become pollinator specialists? *Oikos* **95**: 177–184.

Alcantara, S., de Oliveira, F.B. & Lohmann, L.G. 2013. Phenotypic integration in flowers of Neotropical lianas: diversification of form with stasis of underlying patterns. *J. Evol. Biol.* **26**: 2283–2296.

Armbruster, W.S., Pélabon, C., Bolstad, G.H. & Hansen, T.F. 2014. Integrated phenotypes: Understanding trait covariation in plants and animals. *Philos. Trans. R. Soc. B Biol. Sci.* **369.**

Armbruster, W.S. & Wege, J.A. 2019. Detecting canalization and intra-floral modularity in triggerplant (Stylidium) flowers: correlations are only part of the story. *Annals of Botany* **123**: 355–372.

Baack, E., Melo, M.C., Rieseberg, L.H. & Ortiz-Barrientos, D. 2015. The origins of reproductive isolation in plants. *New Phytol.* **207**: 968–984.

Bank, C., Hermisson, J. & Kirkpatrick, M. 2012. Can reinforcement complete speciation? *Evolution* **66**: 229–239.

Beardsley, P.M. et al. 2003. AFLP phylogeny of *Mimulus* section Erythranthe and the evolution of hummingbird pollination. *Evolution* **57**:1397-1410.

Benitez-Vieyra, S., Pérez-Alquicira, J., Sazatornil, F.D., Domínguez, C.A., Boege, K., Pérez-Ishiwara, R., *et al.* 2019. Evolutionary transition between bee pollination and hummingbird pollination in *Salvia*: comparing means, variances and covariances of corolla traits. *J. Evol. Biol.* 1–11.

Berg, R.L. 1959. A general evolutionary principle underlying the origin of developmental homeostasis. *Am. Nat.* **93**: 103–105.

Berg, R.L. 1960. The ecological significance of correlation pleiades. *Evolution* **4**: 171–180.

Coyne, J.A. & Orr, H.A. 2004. *Speciation*, 1st ed. Sinauer Associates, Sunderland, Massachusetts.

Esteve-Altava, B. 2017. In search of morphological modules: a systematic review. *Biological Reviews* **92**: 1332–1347.

Felice, R.N., Randau, M., & Goswami, A. 2019. A fly in a tube: macroevolutionary expectations for integrated phenotypes. *Evolution* **72**: 2580–2594.

Fenster, C.B., Armbruster, W.S., Wilson, P., Dudash, M.R. & Thomson, J.D. 2004. Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst.* **35**: 375–403.

Fleming, P.A., Hartman Bakken, B., Lotz, C.N. & Nicolson, S.W. 2004. Concentration and temperature effects on sugar intake and preferences in a sunbird and a hummingbird. *Funct. Ecol.* **18**: 223–232.

Forest, F., Goldblatt, P., Manning, J.C., Baker, D., Colville, J.F., Devey, D.S., *et al.* 2014. Pollinator shifts as triggers of speciation in painted petal irises (*Lapeirousia*: Iridaceae). *Ann. Bot.* **113**: 357–371.

Givnish, T.J. et al. (2009) Origin, adaptive radiation and diversification of the Hawaiian lobeliads (Asterales: Campanulaceae). *Proc. Royal Society B* **276**: 407-416.

Goldblatt, P. et al. (1995) Pollination biology of *Lapeirousia* (Iridaceae) in Southern Africa: floral divergence and adaptation for long-tongued fly pollination. *Ann. Miss. Bot. Gar.* **82**: 517-534.

Gómez, J.M., Perfectti, F. & Klingenberg, C.P. 2014. The role of pollinator diversity in the evolution of corolla-shape integration in a pollination-generalist plant clade. *Philos. Trans. R. Soc. B Biol. Sci.* **369**.

Gómez, J.M., Torices, R., Lorite, J., Klingenberg, C.P. & Perfectti, F. 2016. The role of pollinators in the evolution of corolla shape variation, disparity and integration in a highly diversified plant family with a conserved floral bauplan. *Ann. Bot.* **117**: 889–904.

Grant, V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proc. Natl. Acad. Sci.* **91**: 3–10.

Hanley, M.E. *et al.* 2009. Pollination and plant defence traits co-vary in Western Australian *Hakaeas*. *New Phytologist* **182**: 251–260.

Herrera, C.M., Cerdá, X., García, M.B., Guitián, J., Medrano, M., Rey, P.J., *et al.* 2002. Floral integration, phenotypic covariance structure and pollinator variation in bumblebee-pollinated *Helleborus foetidus*. *J. Evol. Biol.* **15**: 108–121.

Hopkins, R. 2013. Reinforcement in plants. *New Phytol.* 197: 1095–1103.

Kay, M.K. et al. (2005) Rapid speciation and the evolution of hummingbird pollination in Neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS. *Am. J. Bot.* **92**:1899–1910.

Kay, K.M. & Sargent, R.D. 2009. The role of animal pollination in plant speciation: integrating Ecology, Geography, and Genetics. *Annu. Rev. Ecol. Evol. Syst.* **40**: 637–656.

Kay, K.M. & Schemske, D.W. 2008. Natural selection reinforces speciation in a radiation of neotropical rainforest plants. *Evolution* **62**: 2628–2642.

Kay, K.M. & Schemske, D.W. 2003. Pollinator assemblages and visitation rates for 11 Species of Neotropical *Costus* (Costaceae). *Biotropica* **35**: 198–207.

Klingenberg, C.P., Badyaev, A.V., Sowry, S.M & Beckwith N.J. 2001. Inferring developmental modularity from morphological integration: analysis of individual variation and asymmetry in bumblebee wings. *Am. Nat.* **157**: 11–23.

Landis, J.B., Bell, C.D., Hernandez, M., Zenil-Ferguson, R., McCarthy, E.W., Soltis, D.E., *et al.* 2018. Evolution of floral traits and impact of reproductive mode on diversification in the Phlox family (Polemoniaceae). *Mol. Phylogenet. Evol.* **127**: 878–890.

Lázaro, A. & Santamaría, L. 2016. Flower-visitor selection on floral integration in three contrasting populations of *Lonicera implexa*. *Am. J. Bot.* **103**: 325–336.

Losos, J.B. 2011. Convergence, adaptation, and constraint. *Evolution* 65: 1827–1840.

Lowry, D.B., Modliszewski, J.L., Wright, K.M., Wu, C.A. & Willis, J.H. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philos. Trans. R. Soc. B Biol. Sci.* 363: 3009–3021.

McDade, L.A. 1992~~.~~ Pollinator relationships, biogeography, and phylogenetics. *BioScience* **42**: 21-26.

Martén-Rodríguez, S., Fenster, C.B., Agnarsson, I., Skog, L.E. & Zimmer, E.A. 2010. Evolutionary breakdown of pollination specialization in a Caribbean plant radiation. *New Phytol.* **188**: 403–417.

Moreira-Hernández, J.I. & Muchhala, N. 2019. Importance of pollinator-mediated interspecific pollen transfer for Angiosperm evolution. *Annu. Rev. Ecol. Evol. Syst.* **50**: 1–27.

Muchhala, N. 2007. Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. *Am. Nat.* **169**: 494–504.

Orr, H.A. & Turelli, M. 2001. The evolution of postzygotic isolation: accumulating Dobzhansky-Muller incompatibilities. *Evolution* **55**: 1085–1094.

Papiorek, S., Junker, R.R., Alves-dos-Santos, I., Melo, G.A.R., Amaral-Neto, L.P., Sazima, M., *et al.* 2016. Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* **18**: 46–55.

Pérez-Barrales, R., Arroyo, J. & Scott Armbruster, W. 2007. Differences in pollinator faunas may generate geographic differences in floral morphology and integration in Narcissus papyraceus (Amaryllidaceae). *Oikos* **116**: 1904–1918.

Pérez, F., Arroyo, M.T.K. & Medel, R. 2007. Phylogenetic analysis of floral integration in *Schizanthus* (Solanaceae): Does pollination truly integrate corolla traits? *J. Evol. Biol.* **20**: 1730–1738.

Proctor, M., Yeo, P. & Lack, A. 1996. *The natural history of pollination*, 1st ed. (T. P. Inc., ed). Portland, Oregon.

Raguso, R.A. 2004. Flowers as sensory billboards: progress towards an integrated understanding of floral advertisement. *Curr. O. Plant Biol.* **7**: 434–440.

Ramírez-Aguirre, E., Martén-Rodríguez, S., Quesada-Avila, G., Quesada, M., Martínez-Díaz, Y., Oyama, K., *et al.* 2019. Reproductive isolation among three sympatric *Achimenes* species: pre- and post- pollination components. *Am. J. Bot.* **106**: 1021–1031.

Ramírez-Roa, M.A. de la P. 1987. Revisión de *Achimenes* (Gesneriaceae). Tesis de de la Facultad de Ciencias. Universidad Nacional Autónoma de México.

Reich, D., Berger, A., von Balthazar, M., Chartier, M., Sherafati, M., Shönenberger, J., Manafzadeh, S. & Steadler, Y. M. 2020. Modularity and the evolution of floral shape: the role of function, development and spandrels in *Erica*. *New Phytol*. **226**: 267–280.

Reynolds, R.J., Dudash, M.R. & Fenster, C.B. 2009. Multiyear study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated *Silene virginica*. *Evolution* **64**: 358–369.

Rieseberg, L.H. & Willis, J.H. 2007. Plant speciation. *Science* **317**: 910–914.

Rosas-Guerrero, V., Aguilar, R., Martén-Rodríguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J.M., *et al.* 2014. A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecol. Lett.*, doi: 10.1111/ele.12224.

Rosas-Guerrero, V., Quesada, M., Armbruster, W.S., Pérez-Barrales, R. & Smith, S.D.W. 2011. Influence of pollination specialization and breeding system on floral integration and phenotypic variation in *Ipomoea*. *Evolution* **65**: 350–364.

Schiestl, F.P. & Johnson, S.D. 2013. Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* **28**: 307–315. Elsevier Ltd.

Servedio, M.R. & Noor, M.A.F. 2003. The role of reinforcement in speciation: theory and data. *Annu. Rev. Ecol. Evol. Syst.* **34**: 339–364.

Smith, S.D. et al. 2008. The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution* **62**: 793-806.

Smith, S.D. 2016. Pleiotropy and the evolution of floral integration. *New Phytol.* **209**:80–85.

Smith, S.D.W. 2010. Using phylogenetics to detect pollinator-mediated floral evolution. *New Phytol.* **188**: 354–363.

Smith, S.D.W & Kriebel, R. 2018. Convergent evolution of floral shape tied to pollinator shifts in *Iochrominae* (Solanaceae). *Evolution* **72**: 688–697.

Sobel, J.M. & Chen, G.F. 2014. Unification of methods for estimating the strength of reproductive isolation. *Evolution* **68**: 1511–1522.

Sobel, J.M., Chen, G.F., Watt, L.R. & Schemske, D.W. 2010. The biology of speciation. *Evolution* **64**: 295–315.

Stebbins, G.L. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. *Annu. Rev. Ecol. Syst.* **1**: 307–326.

Strauss, S.Y. & Whittall, J.B. 2006. Non-pollinator agents of selection. In: Ecology and Evolution of Flowers (L. D. Harder & S. C. H. Barrett, eds.), pp. 120–135. Oxford University Press, New York.

Tripp, E.A. & Manos, P.S. 2008. Is floral specialization an evolutionary dead-end? Pollination system transitions in *Ruellia* (Acanthaceae). *Evolution* **62**: 1712–1737.

Valente, L.M. et al. 2012. Did pollination shifts drive diversification in Southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *Am. Nat.***180**: 83-98.

van der Niet, T. & Johnson, S.D. 2012. Phylogenetic evidence for pollinator- driven diversification of angiosperms. *Trends Ecol. Evol.* **27**: 353–361.

Wessinger, C.A., Hileman, L. & Rausher M.D. 2014. Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Phil. Trans. R. Soc. B.* **369**: 20130349.

Wessinger, C.A. & Hileman L.C. 2016. Accessibility, constraint, and repetition in adaptive floral evolution. *Dev. Biol.* 419: 175–183.

Whittall, J.B. & Hodges, S.A. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**: 706–710.

Wilson, P., Wolfe, A.D., Armbruster, W.S., Thomson, J.D. & Wilson, P. 2007. Constrained lability in floral evolution: counting convergent origins of hummingbird pollination in *Penstemon* and *Keckiella*. *New Phytol.* **176**: 883–890.

Wózniak, N.J. & Sicard A. 2018. Evolvability of flower geometry: convergence in pollinator-driven morphological evolution of flowers. *Sem. Cell & Dev.* **79**: 3–15.

Yuan, Yao-Wu, Byers, K. JRP & Bradshaw H.D. 2013. The genetic control of flower-pollinator specificity. *Curr. Op. Plant Biol.* **16**: 422–428.