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Interspecific Pollen Transfer, Gene Flow, and Speciation in Bat-Pollinated Burmeistera H. Karst. & Triana (Campanulaceae: Lobelioideae)

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Interspecific Pollen Transfer, Gene Flow, and Speciation in Bat-Pollinated *Burmeistera* H. Karst. & Triana (Campanulaceae: Lobelioideae)

By

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Abstract

The process of speciation is one central subject of study in evolutionary biology, as it is the path through by which biological diversity arises on the planet. The remarkable evolutionary success of flowering plants is thought to have been driven in no small part by their mutualistic interactions with animal pollinators, which provide pollen and thus gene transport between individuals, populations, and even incipient species on already decidedly distinct evolutionary trajectories. Here, I examine the interplay between interspecific pollen transfer by shared pollinators, gene flow patterns, and the evolution of reproductive isolation in the young rapid radiation of Neotropical bat-pollinated bellflowers in the genus *Burmeistera* (Campanulaeae: Lobelioideae). In Chapter 1, I conducted an extensive review of the pollination and plant speciation literature to highlight the evolutionary consequences of pollinator-mediated interspecific pollen transfer in angiosperms. I showed that pollen transfer between species has profound consequences for the evolution of floral traits, reproductive isolation barriers, and patterns of gene flow during speciation. Importantly, I pointed to a strikingly common, yet not sufficiently discussed, pattern evident in the literature: that interactions via pollen transfer between closely-related plant species often result in asymmetries of reproductive isolation between them. Whether such asymmetries in pollen flow generate differential fitness consequences for interacting plant species was the subject of Chapter 2. In this chapter, I studied how patterns of simulated heterospecific pollen deposition affect fruit and seed production in two sympatric *Burmeistera* species pairs that experience asymmetric pollen transfer among them by shared bat pollinators. I found support for the idea that asymmetric pollen flow results in the evolution of strong barriers against heterospecific pollen in those species that frequently receive pollen from their relatives, with species that are less exposed exhibiting comparatively weaker barriers. In Chapter 3, I studied patterns of interspecific pollen transfer and introgressive gene flow in three communities of sympatric bat-pollinated *Burmeistera* to examine a possible relationship between pollen and gene flow during the evolution of the group. Although interspecific pollen transfer was prevalent among our *Burmeistera* communities and

involved all study species, we did not detect a significant signal of past introgression between species suggesting that reproductive isolation at the gametic or postzygotic stages is sufficient to prevent interspecific gene flow. These results show that rapid diversification in the absence of obvious shifts in pollinators can still lead to the successful establishment of barriers to gene flow between sympatric species. Finally, in Chapter 4 I assembled a dataset of multiple pre- and post-pollination barriers for 11 species pairs of *Burmeistera* along a continuum of evolutionary divergence. I found that mean reproductive barrier strength was higher for post-pollination barriers compared to pre-pollination isolation, yet because of the sequential nature of reproductive isolation both stages have similar relative contributions to the observed levels of total isolation among pairs. Lastly, using a robust dated phylogeny for *Burmeistera* I uncovered a linear positive relationship between post-pollination barriers and time since divergence among pairs, whereas such relationship was not found for pre-pollination isolation. Together, these results suggest that post-pollination isolation has been very important to prevent gene flow and promote divergence during the diversification of *Burmeistera*, and that current floral differences conferring pre-pollination isolation have evolved more recently to prevent reproductive interference via interspecific pollen transfer after secondary contact. Much attention has been paid to how specialization to different pollinator species contributes to diversification by promoting reproductive isolation. However, less attention has been given to how interactions mediated by the very pollen pollinators carry may contribute to flower evolution, genetic exchange, and reproductive isolation during speciation. This dissertation is my contribution to alleviate this oversight, by showing how the extraordinary radiation of *Burmeistera* has indeed proceeded while faithfully upholding their close partnership with their furry nectar-seeking bat friends.

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CHAPTER 1.

Importance of pollinator-mediated interspecific pollen transfer for angiosperm evolution

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Abstract

Understanding how pollen moves between species is critical to understanding speciation, diversification, and evolution of flowering plants. For co-flowering species that share pollinators, competition through interspecific pollen transfer (IPT) can profoundly impact floral evolution, decreasing female fitness via heterospecific pollen deposition on stigmas and male fitness via pollen misplacement during visits to heterospecific flowers. The pollination literature demonstrates that such reproductive interference frequently selects for reproductive character displacement in floral traits linked to pollinator attraction, pollen placement, and mating systems, and has also revealed that IPT between given pairs of species is typically asymmetric. More recent work is starting to elucidate its importance to the speciation process, clarifying the link between IPT and current and historical patterns of hybridization, the evolution of phenotypic novelty through adaptive introgression, and the rise of reproductive isolation. Our review aims to stimulate further research on IPT as a ubiquitous mechanism that plays a central role in angiosperm diversification.

Keywords: reproductive interference, plant competition, hybridization, reproductive isolation, floral evolution, plant speciation

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1. INTRODUCTION

Pollen grains from approximately 300,000 species worldwide, corresponding to 87.5% of angiosperms, are transported by a variety of animal pollinators (Ollerton et al. 2011). When pollinators alternate foraging visits between co-flowering, co-occurring plant species, pollen may be transferred interspecifically (Morales & Traveset 2008). Interspecific pollen transfer (IPT) has long been recognized in the pollination literature as a form of reproductive interference; a type of competitive interaction that decreases fitness for at least one of the interacting species (Campbell 1985, Mitchell et al. 2009, Waser 1978a, Waser 1983, Rathcke 1983). This fitness decrease can be due to either heterospecific pollen deposition on stigmas, which can reduce seed set by clogging stigmas or usurping ovules (Ashman & Arceo-Gómez 2013, Jakobsson et al. 2008, Briggs et al. 2015), or to pollen misplacement during foraging on heterospecific flowers, which will reduce successful pollen export to conspecific stigmas (Minnaar et al. 2019, Muchhala & Thomson 2012, Thomson et al. 2018). IPT dictates patterns of interspecific gene flow when species are closely related (Campbell et al. 2002, Harder et al. 1993, Kay 2006, Natalis & Wesselingh 2012a), and thus its study is also critical to understanding plant diversification in terms of the speciation process, reproductive isolation, adaptive introgression, and hybridization.

A decade ago, Morales & Traveset (2008) contributed the first and only comprehensive review on IPT, carefully laying out evidence for the occurrence of IPT in nature and the expected ecological and evolutionary consequences. Prior to this seminal publication, IPT tended to receive less attention than other forms of competition between co-flowering plants, such as competition for pollinator attraction (Ashman & Arceo-Gómez 2013, Muchhala & Thomson 2012, Mitchell et al. 2009). However, pollinator sharing and generalization are widespread in pollination networks (Arceo-Gómez et al. 2016a, Bascompte et al. 2006), and multiple recent community-level studies to show that IPT is more common than previously thought (Arceo-Gómez et al. 2018, Fang & Huang 2013, Johnson & Ashman 2019, Tur et al. 2016). In addition, IPT interactions have

recently been highlighted as one of the major sources of pollen loss along the paternity pathway from pollen production to ovule fertilization (Minnaar et al. 2019), underscoring its importance for plant reproduction and floral evolution. This growing recognition has stimulated a burgeoning literature, including studies on the mechanics of IPT in terms of how the presence of competitors affects pollen export and receipt (Flanagan et al. 2009, Minnaar et al. 2019, Muchhala & Thomson 2012, Thomson et al. 2018), and the evolutionary consequences of IPT in terms of selection for specialization on pollinators (Armbruster et al. 2014, Muchhala et al. 2010), character displacement in floral phenotype (Eaton et al. 2012, Grossenbacher & Stanton 2014, Muchhala et al. 2014), and the evolution of mating systems (Briscoe Runquist & Moeller 2014, Randle et al. 2018). Importantly, the movement of pollen between species and its evolutionary costs have been repeatedly shown to be highly asymmetric (Briscoe Runquist 2012, Natalis & Wesselingh 2012a, Randle et al. 2018). Inspired by this intensified interest on IPT as a ubiquitous process in nature, here we review our current understanding of its implications for angiosperm ecology and evolution.

Our first main goal is to present a critical synthesis of our current understanding of IPT and its consequences. In Section 2 we review the fitness costs of pollen misplacement and heterospecific pollen deposition, and in Section 3 we explore the implications of these costs for floral divergence, specialization, and mating system evolution. Our second main goal is to explore the intersection between the pollination ecology perspective of IPT and the evolutionary implications of IPT in terms of how it affects gene flow during early plant diversification. In Section 4 we present outline the expected outcomes of pollen transfer between a pair of species based on the time since they shared a common ancestor, and the consequences for reproductive isolation and the transfer of adaptive genetic variation. We conclude by emphasizing the emerging patterns in an evolutionary context and highlighting underexplored issues particularly deserving of future research.

2. EFFECTS OF INTERSPECIFIC POLLEN TRANSFER ON FLORAL FITNESS

From the plant perspective, the fitness of a flower is maximized by increasing pollen dispersal to conspecific flowers and by ensuring the receipt of sufficient conspecific pollen to fertilize its ovules (Mitchell et al. 2009, Morales & Traveset 2008). These components of floral fitness correspond to the male and female functions, respectively. The degree to which a pollinator maximizes male and female fitness is termed pollinator effectiveness, and it can be further subdivided into quantity and quality components (Ne'eman et al. 2010). The quantity component refers to the number of visits a pollinator makes, while quality refers to the amount of pollen that is transported per visit, as well as the genetic attributes of this pollen (in terms of the diversity of sires and the amount of outcross vs. self pollen; Mitchell et al. 2009, Ne'eman et al. 2010). Both components of pollinator effectiveness are typically thought to be determined by factors intrinsic to the vector, including foraging behavior, floral fidelity, visitation behavior, and visitation rates (Armbruster 2014, Flanagan et al. 2009, Muchhala et al. 2009, Ne'eman et al. 2010), but this perspective overlooks the fact that pollinator effectiveness may fundamentally change in the presence of competitor plant species (an extrinsic factor) if this leads to increased heterospecific pollen transfer (negatively affecting female fitness) and/or pollen misplacement (negatively affecting male fitness; Mitchell et al. 2009, Muchhala & Thomson 2012). Such effects may be highly asymmetric, affecting one competitor more than the other, due to idiosyncrasies of pollinator preference, floral morphology, spatial arrangement, species abundances, and post-pollination reproductive barriers (Muchhala & Thomson 2012, Natalis & Wesselingh 2012, Thomson et al. 2018). In the following subsections we review evidence for negative effects of pollen misplacement on male fitness followed by negative effects of heterospecific pollen deposition on female fitness.

2.1. Pollen Misplacement

Male fitness in plants requires efficient pollen transport from the anthers where it is produced to conspecific stigmas where it can germinate, produce a pollen tube, reach the ovary, and deploy the sperm cells that will ultimately effect ovule fertilization (Minnaar et al. 2019, Mitchell et al. 2009, Morales & Traveset 2008). Mounting evidence shows that pollen loss during transport is arguably the largest factor affecting male fitness, as the vast majority of pollen never reaches conspecific stigmas (Minnaar et al. 2019). Throughout this paper, we use pollen misplacement to refer specifically to competitive costs due to the loss of pollen during visits to competitor species; this includes pollen deposited on foreign stigmas or other plant structures, as well as pollen lost from pollinators' bodies due to passive detachment or active grooming (Muchhala & Thomson 2012). We prefer pollen misplacement to 'conspecific pollen loss' (Morales & Traveset 2008) because of the referential difficulties of the latter term; pollen lost during visits to foreign flowers is neither conspecific to that flower nor to the source flower (it was produced by the source flower, thus is not conspecific to it; see Muchhala & Thomson 2012)

A critical first step in the pathway to paternity which can have important implications for pollen misplacement involves the deposition of pollen on pollinator's bodies (Minnaar et al. 2019). The interaction between plant traits, including its morphology (e.g. anther size and orientation, corolla constriction, tube length) and the nature of its floral rewards (e.g. position in the flower and quantity), and pollinator traits (including size, shape, and visitation behavior) together determine the amount of pollen placed, its position on the pollinator's body, and the total area it covers (Armbruster et al. 2009a, Huang & Shi 2013, Muchhala 2007). Two co-flowering plant species that place pollen in the same region of a pollinator's body will be at risk of losing pollen every time the vector misplaces it onto the reproductive organs of its competitor (Muchhala & Potts 2007, Muchhala & Thomson 2012, Natalis & Wesselingh 2012a). For a more

thorough discussion of intra- and interspecific competition for pollen placement on pollinator bodies we refer readers to the excellent review by Minnaar et al. (2019).

Even when pollen is deposited on and picked up from different portions of pollinator's bodies, it may still be lost during visits to competitor flowers (Flanagan et al. 2009, Muchhala & Thomson 2012). For example, Murcia & Feinsinger (1996) found no effect of floral morphological similarity (which corresponds with overlapping pollen placement) on pollen losses by foraging hummingbirds alternating between competitor flowers, but still found that visits to competitors decreased the pollen transferred to conspecific stigmas as much as 76%. Most of this pollen loss appeared to be due to corollas of competitor flowers scraping pollen off of the birds' bills (Murcia & Feinsinger 1996). Another innovative study showed that increased grooming frequency by bumblebee pollinators during visits to the invasive competitor Lythrum salicaria (Lythraceae) was the main contributor to pollen misplacement for Mimulus ringens (Phrymaceae; Flanagan et al. 2009). Very little pollen was transferred to heterospecific stigmas, but pollen misplacement due to grooming while visiting competitor flowers was sufficient to limit seed set of M. ringens, showing that male fitness costs can carry over and depress female fitness of a population as well (Flanagan et al. 2009). Finally, Muchhala & Thomson (2012) found that while competitor species with similar sites of pollen placement on bat's bodies suffered the greatest pollen losses, all pairs of species suffered significant amounts of pollen misplacement relative to the amount of pollen transferred without intervening visits to a competitor, demonstrating the importance of losses from pollinators bodies due to passive detachment or active grooming (Fig. 1). Regardless of how exactly pollen is misplaced, studies such as those mentioned above and others in natural and experimental populations show that pollen misplacement can often entail larger overall fitness losses than those incurred through heterospecific pollen deposition (Campbell & Motten 1985, Muchhala & Thomson 2012, Thomson et al. 2018). In spite of this, male floral fitness and pollen misplacement have been much less explored than heterospecific

pollen deposition, likely due to the difficulties associated with accurately tracking pollen grains' fate and/or distinguishing between pollen from closely-related species (Minnaar et al. 2019, Morales & Traveset 2008). Fortunately, in the last decade powerful methods of pollen tracking and identification have emerged, such as individual grain genotyping (e.g. Hasegawa et al. 2015) and bio-labeling (Minnaar & Anderson 2018), which should greatly facilitate the study of male fitness, competition for pollination, and floral evolution (Minnaar et al. 2019).

We know very little about the magnitude and prevalence of pollen misplacement in nature, but recent evidence shows that it can be as extensive and common as heterospecific pollen deposition. One detailed study on the structure of a pollen transfer network of 57 species from an alpine community in China revealed that plant species exported pollen to stigmas of 5.5 (± 5.4 SD) other species on average, and received pollen in their stigmas from 7.2 (± 5.0 SD) other species (Fang & Huang 2013). Interestingly, the number of recipient species per donor species was positively correlated with the total number of pollen grains exported, as were the number of donor species per recipient species and the total number of heterospecific pollen grains received in stigmas (Fang & Huang 2013). In other words, most species either suffered extensive pollen misplacement, experienced high rates of heterospecific pollen deposition from a diversity of sources, or had a minor participation in the network overall. These results and those from other IPT network studies typically show that separate subsets of species regularly experience high rates of pollen misplacement or of heterospecific pollen deposition (Arceo-Gómez et al. 2016a, Fang & Huang 2013, Johnson & Ashman 2019, Tur et al. 2016). However, because they only use stigmatic loads to build IPT networks, these studies underestimate the magnitude of pollen misplacement as they do not account for passive or active pollen detachment during the intervening visits (e.g. Murcia & Feinsinger 1996). Overall, the imbalance in the amount of research on pollen misplacement versus heterospecific pollen deposition has precluded a full understanding of the importance of IPT interactions in nature.

2.2. Heterospecific Pollen Deposition

In contrast to the male function, the fitness costs of heterospecific pollen deposition to female function are much better understood. As with pollen misplacement, the extent to which a species may experience heterospecific pollen deposition depends on spatial and temporal flowering overlap with competitors, the degree of pollinator sharing (simultaneously determined by plant and pollinator traits influencing attraction and pollen deposition/pickup from the pollinator bodies), relative floral abundances, pollinator preference, and visitation behavior (Arceo-Gómez & Ashman 2014, Mitchell et al. 2009, Morales & Traveset 2008, Thomson et al. 2018). In combination, all of these factors determine the quantity and diversity of foreign pollen a flower receives. Below, we review the cascade of negative effects foreign pollen may have on female fitness, and then review our understanding of how the quantity and diversity of these foreign pollen loads modulate these negative effects.

Following the arrival of foreign pollen on a stigma, the first potential negative effects occur on the stigmatic surface. Foreign grains may interact with conspecific grains or with the stigma itself, interfering with conspecific pollen adhesion and germination (Ashman & Arceo-Gómez 2013, Brown & Mitchell 2001). Studies that applied foreign pollen either before, after, or at the same time as conspecific pollen demonstrate the importance of timing: while several studies found that seed set was only decreased when the foreign pollen was applied beforehand (Caruso & Alfaro 2000, Waser & Fugate 1986, Kohn & Waser 1985), one study found that applying foreign pollen before or after had no effect, and seed set was only decreased when foreign and conspecific were applied together (Bruckman & Campbell 2016). The mechanisms by which foreign pollen affects conspecific pollen adhesion and germination can vary, and may include stigma clogging (Galen & Gregory 1989), foreign pollen allelopathy (Thomson et al. 1981, Murphy and Aarssen 1995), induction of the mechanical closure of the stigma (Waser &

Fugate 1986), or triggering incompatibility reactions in the stigma surface that also impact conspecific grains (reviewed in Ashman & Arceo-Gómez 2013).

A second set of negative effects can occur if the foreign pollen germinates and forms pollen tubes. This is particularly likely for more closely-related species, as they may have similar pollen-pistil compatibility. The foreign pollen tubes may negatively impact seed set through stylar clogging as they physically crowd the stylar tissue. This idea makes intuitive sense, and is supported by the fact that several hand-crossing studies using a single self-incompatible species show that mixing incompatible (i.e. self) pollen with compatible pollen reduces seed set via stylar clogging (Shore & Barrett 1984, Palmer et al. 1989, Scribailo & Barrett 1994). However, we are not aware of a study clearly showing stylar clogging in crosses between pairs of species. The strongest evidence for such an effect involves crosses between the congeners Impatiens capensis and I. pallida, which found seed set was only reduced when I. capensis was the recipient, and that I. pallida pollen tubes can reach the ovaries in I. capensis styles, while I. capensis pollen fails to adhere to I. pallida stigmas (Randall & Hilu 1990). This would seem to implicate stylar clogging, but does not rule out that negative effects may be due solely to interactions on the stigmatic surface.

Finally, assuming it successfully germinates on the stigma and forms a pollen tube capable of reaching the ovules, foreign pollen from closely-related species may release sperm and fertilize ovules, causing the recipient plant to waste precious maternal resources (Jakobsson et al. 2008). Such usurped ovules are no longer available for conspecific fertilization, a fitness cost termed ‘interspecific seed discounting’ (Burgess et al. 2008), and may lead to seed or whole fruit abortion (Fishman & Wyatt 1999, Montgomery et al. 2010, Wang & Cruzan 1998, Wolf et al. 2001), seed germination failure (Natalis & Wesselingh 2012b), or the production of unfit or sterile offspring (Goodwillie & Ness 2013).

Interestingly, for crosses between a given pair of species, the relative ability of one species to germinate, form pollen tubes, and fertilize ovules of the other is typically significantly asymmetric (Tiffin et al. 2001). Two main explanations have been put forward to explain such asymmetry. First, it may be due to idiosyncratic differences in the mechanisms plants use to suppress heterospecific pollen that reach their stigmas. In most plants, such incompatibility reactions result in conspecific pollen precedence (Howard 1999); i.e.,conspecific pollen enjoy superior germination, pollen tube growth rates, or ability to enter the ovary and fertilize the ovules relative to foreign pollen (Lyu et al. 2016, Montgomery et al. 2010); however the specific stages that this occurs or mechanisms used to suppress growth often differs between species (Figueroa-Castro & Holtsford 2009, Fishman et al. 2008, Harder et al. 1993, Lyu et al. 2016, Montgomery et al. 2010, Randall & Hilu 1990). A second explanation for asymmetry in crossing barriers is that they are due to difference in style lengths. Typically, the size of a plant's pollen grains correlates with maximum pollen tube size, which correlates with style length for that species (Brothers & Delph 2017, Carney et al. 1996). This pattern can lead to smaller-grained pollen from short-styled species not being able to reach the ovary and effect fertilization in flowers of long-styled species, while crosses in the opposite can occur unimpeded (Carney et al. 1996, Diaz & Macnair 1999, Kay 2006, Wolf et al. 2001).

Now that we have outlined the cascade of negative effects heterospecific pollen can have on female fitness, we will turn to how quantity and diversity of heterospecific pollen loads can modulate these effects. First, heterospecific pollen has been found to account for up to 74% of total pollen receipt in nature (Arceo-Gómez et al. 2016a, Ashman & Arceo-Gómez 2013); how does increasing heterospecific pollen quantity affect the fitness costs? Unfortunately few studies directly address this question; most examine heterospecific pollen deposition by applying a 50:50 ratio of conspecific to heterospecific pollen to stigmas (Ashman & Arceo-Gómez 2013). In one study that varied this ratio, no amount of heterospecific pollen from invasive nightshade Solanum

elaeagnifolium (Solanaceae) decreased seed production in the poppy-relative Glaucium flavum (Papaveraceae) as long as some conspecific pollen was present (Papaveraceae; Tscheulin et al. 2009). In four other cases involving pairs of closely-related hybridizing species, the relative proportion of heterospecific pollen was inversely correlated with seed production, although the strength of this relationship varied across the different recipient species (Harder et al. 1993, Montgomery et al. 2010, Ramsey et al. 2003, Wang & Cruzan 1998). In an additional study, the proportion of heterospecific pollen did not affect total seed set but predicted the proportion of hybrid seeds produced (Alarcon & Campbell 2000). Finally, we are aware of only one study to examine effects of variable amounts of heterospecific pollen on seed set in natural settings (rather than experimental hand-pollinations): for the herb Delphinium barbeyi, receipt of greater amounts of heterospecific pollen dampened the positive interaction between conspecific pollen receipt and seed set (Briggs et al. 2015). Thus, overall, the limited research to date tends to support the conclusion that greater amounts of heterospecific pollen lead to lower successful conspecific seed set.

Similar to the above question about the effects of quantity, how does diversity of heterospecific pollen loads affect the fitness costs? We know of only one study that directly addressed this question. For the monkey-flower Mimulus guttatus (Phrymaceae), seed set decreased with increasing number of foreign pollen donor species, although the effect size of this pattern varied depending on donor identity (Arceo-Gómez & Ashman 2011). Pollen from one species, the sunflower Helianthus exilis (Asteraceae), was capable of reducing M. guttatus seed set by the same magnitude as its congener M. nudatus, and also equaled the combined effect from a mixture of M. nudatus and the mint-relative Stachys albens (Lamiaceae; Arceo-Gómez & Ashman 2011). The authors hypothesized that the strong negative effect H. exilis had on M. guttatus seed set was due to a combination of the large size and spiny surface of its pollen grains, its ability to germinate in M. guttatus stigmas, and possibly additional allelopathic effects by

negatively affecting conspecific pollen germination. M. nudatus, on the other hand, reduced seed production at a later stage by usurping ovules and promoting seed abortion. The negative effects of S. albens on M. guttatus seed set were weak unless in combination with pollen from the other two competitors (Arceo-Gómez & Ashman 2011). Although this remains the only study of its kind, it suggests that female fitness responses to diverse heterospecific pollen loads may be highly species- and context-specific. Given the extreme variability in amount of foreign pollen receipt found within and among plant communities (Arceo-Gomez et al. 2016a, Fang & Huang 2013, Johnson & Ashman 2019, McLernon et al. 1996, Tur et al. 2016), this represents a much-needed avenue for future research.

3. EVOLUTIONARY RESPONSES TO INTERSPECIFIC POLLEN TRANSFER

Angiosperms have evolved a wide range of strategies to reduce the impact of IPT on fitness, which can be categorized into three main types. The first involves adaptations to prevent IPT from occurring in the first place (pre-pollination isolation), which can reduce both pollen misplacement and heterospecific pollen deposition, thus improving male and female fitness (Armbruster et al. 1994, Kay et al. 2018, Muchhala et al. 2014). The second involves adaptations to counteract foreign pollen germination and performance after heterospecific pollen arrives on stigmas (gametic isolation), which limits negative effects on female fitness (Arceo-Gómez et al. 2016b, Kay & Schemske 2008, Natalis & Wesselingh 2012b). A third type of evolutionary response to IPT involves an increase in autonomous self-pollination rates, which allows conspecific (selfed) seed set even when large amounts of foreign pollen are deposited (Randle et al. 2018, Briscoe Runquist & Moeller 2014, Smith & Rausher 2008).

Before discussing these three responses to IPT, we would first like to clarify pertinent terminology. By pre-pollination isolation, we mean any reproductive barriers that act to reduce

IPT, and thus arrival of foreign pollen to stigmas. Gametic isolation refers to barriers that occur as the gametes interact, from the point that foreign pollen arrives to stigmas up until it fertilizes ovules (Coyne & Orr 2004). Both of these are forms of prezygotic isolation, while any barriers that serve to reduce gene flow after ovules are fertilized are termed postzygotic isolation. It is important to note that we still consider pre-pollination and gametic barriers as forms of reproductive isolation, regardless of whether gene flow can actually occur between the pair of species, because they will still serve to limit reproductive interference. Any evolutionary increases in prezygotic barrier strength in response to pollen transfer between species in sympatry is termed reproductive character displacement, whether or not the species are already fully reproductively isolated through post-pollination barriers, while a special form of reproductive character displacement termed reinforcement occurs when natural selection favors such increases in barrier strength in the face of ongoing gene flow (Beans 2014, Hopkins 2013, Kay & Schemske 2008). In the following three subsections, we explore how plants may respond to competition through interspecific pollen transfer, with or without accompanying gene flow, through evolutionary increases in pre-pollination isolation, gametic isolation, or selfing rates.

3.1. Pre-Pollination Isolation

When IPT occurs, selection may favor divergence in several aspects of floral phenotype to increase pre-pollination isolation, thus reducing the fitness costs arising from pollen misplacement and heterospecific pollen deposition. First, the competing species may diverge in phenology, flowering at different times of the day or of the year, which is termed temporal isolation (Borchsenius et al. 2016, Hipperson et al. 2016, Martin & Willis 2007, Paudel et al. 2018, Waser 1978b, Yang et al. 2007, Zhang et al. 2016). In such instances, if flowering overlap is not completely eliminated, the later-flowering species might still experience low but detectable fitness costs when its first-flowering individuals are at a large numerical disadvantage versus

earlier-flowering competitors (e.g. Waser 1978b). Similarly, among hybridizing species, the later-flowering species might suffer asymmetric hybridization from its earlier-flowering relative (Martin & Willis 2007, Zhang et al. 2016). To date, no studies have found support for either reproductive character displacement or reinforcement of temporal isolation when comparing sympatric and allopatric populations (Christie & Strauss 2018, Kay 2006, Paudel et al. 2018), but it is possible that this is often an initial step on secondary contact and that, once additional barriers to IPT evolve, flowering time differences quickly relax (Christie & Strauss 2018).

A second response to IPT, termed floral isolation, involves diverging in the use of pollinators to reduce the amount of pollen they transfer between species. Floral isolation can be divided into two subcomponents: ethological isolation, which involves differences in floral traits affecting pollinator preference and thus reducing interspecific pollinator movements, and mechanical isolation, which involves differences in traits that influence the mechanical fit between flower and pollinator during visits (Grant 1994, Schiestl & Schlüter 2009). For the former, the most direct way to achieve ethological isolation is for competing species to specialize on different pollinator types by diverging in attraction traits or in morphology to restrict access to rewards, thus eliminating interspecific pollinator movements (Muchhala et al. 2010, Rodríguez-Gironés & Santamaría 2007). A less obvious way to achieve ethological isolation involves increasing floral constancy, or the degree to which individual pollinators stick to one flower type during foraging bouts instead of switching between types (Waser 1986, Amaya-Márquez 2009). This can lead to, for example, a bumblebee being classified as generalized on a species or colony level despite individuals being highly specialized to different species of flowering plant, and thus not contributing to competition via IPT (Oyama et al. 2010). There are three proposed mechanisms by which shifts in floral traits could improve constancy. First, if accessing nectar rewards is complicated, this may encourage sticking with one flower type due to constraints on the ability to learn and remember how to manipulate multiple types (Chittka et al. 1999, Gegear

& Laverty 2005, Laverty 1994). Second, differences in floral traits could reinforce search images used to locate flowers during foraging (Heinrich 1975, Wilson & Stine 1996, Goulson 2000). For instance, bat-pollinated *Burmeistera* flowers present extreme interspecific variation in the size, shape, and orientation of the leaf-like calyx lobes at the base of their flowers, which likely reflect echolocation calls very differently (Muchhala 2006); when multiple species co-occur, this may encourage individual bats to learn and stick with a single species. A third mechanism to encourage floral constancy involves differences that encourage and reinforce social hierarchies among pollinators that aggressively defend resources, causing dominant individuals to stick with different flowers than subordinate individuals (Muchhala et al. 2014). Experiments with hummingbirds and artificial flowers in flight cages support this idea; when provided with two flower types with either high or low nectar rewards, dominant male and subordinate female *Anthracothorax jugularis* visited both types indiscriminately, but when the same types had different colors, the sexes partitioned the resource, with males sticking with the high-reward flowers and vice-versa (Temeles et al. 2017). Although more work is needed to understand the extent to which these three mechanisms contribute to floral constancy, all three lead to similar patterns, in that they all favor diverging from sympatric competitors in floral traits (e.g., De Jager et al. 2011, Weber et al. 2018, Takahashi et al. 2016).

Mechanical isolation, the other subcomponent of floral isolation, can be achieved through changes in the length, shape, or orientation of the floral reproductive parts, or of other aspects of floral morphology that affect the pollinator positioning during visits, causing divergence in pollen placement. (Armbruster et al. 1994, Huang & Shi 2013, Huang et al. 2015, Kay et al. 2018, Muchhala & Potts 2007). In fact, many studies on IPT and floral evolution have shown that small trait adjustments can have large impacts on pollinator efficiency in terms of pollen transport and delivery (Castellanos et al. 2003, More et al. 2007, Muchhala & Potts 2007). However, it is important to note that even a total shift in pollen placement on shared pollinators may fail to

eliminate male fitness costs from pollen misplacement, because as long as pollinators move between species pollen may still be lost to grooming or may be scraped off pollinator's body during intervening visits to competitors (Flanagan et al. 2009, Muchhala & Thomson 2012); thus ethological isolation is more effective at preventing pollen misplacement. On the other hand, mechanical isolation can effectively eliminate costs to female fitness from heterospecific pollen deposition.

3.2. Gametic Isolation

The costs to female fitness from IPT can be reduced by various forms of gametic isolation, including stigma incompatibility and suppression of pollen tube growth rate (Ashman & Arceo-Gómez 2013). Stigmas can evolve to increase incompatibility with foreign pollen by altering stigma structure (Arceo-Gómez & Ashman 2011, Caruso & Alfaro 2000), the chemical composition of stigma exudates (Kay & Schemske 2008), or the factors controlling pollen recognition and self-incompatibility (Bedinger et al. 2017). These three mechanisms need not be mutually exclusive, and they usually suffice to prevent germination of pollen among distantly-related species (but see Arceo-Gómez & Ashman 2011). Although few studies have determined the precise isolating mechanisms operating at the stigma surface (Bedinger et al. 2017), the importance of the self-incompatibility pathway can be seen in instances of asymmetric rejection of pollen from self-compatible species on stigmas of self-incompatible relatives (Ashman & Arceo-Gómez 2013, Brandvain & Haig 2005; see Section 3.3).

Differential pollen tube performance in the style constitutes the next main form of gametic isolation and is usually found only among close relatives, given that pollen from more distantly-related species typically fails to germinate. This barrier occurs through two main mechanisms. The first, termed conspecific pollen precedence (Howard 1999), results from

incompatibility reactions elicited by foreign pollen such that conspecific pollen performs better in terms of germination, pollen tube growth rates, access to the ovary, and ovule fertilization relative to foreign pollen (Lyu et al. 2016, Montgomery et al. 2010). Because these various ways in which foreign pollen is suppressed can differ between pairs of closely-related species, there is often asymmetry across pairs in pollen tube performance and/or hybridization (Figueroa-Castro & Holtsford 2009, Fishman et al. 2008, Harder et al. 1993, Lyu et al. 2016, Montgomery et al. 2010; but see Alarcon & Campbell 2000, Natalis & Wesselingh 2012b). The second main mechanism for gametic isolation involves a mismatch between host style length and foreign pollen grain size. Because grain size often determines the maximum pollen tube length it can attain (Brothers & Delph 2017, Carney et al. 1996), smaller-grained pollen from short-styled species often cannot effect fertilization in long-styled species, while the opposite can occur unimpeded (Carney et al. 1996, Diaz & Macnair 1999, Kay 2006, Wolf et al. 2001).

3.3. Evolution of Mating Systems

In self-compatible plant populations, the mating system of a particular population is defined as the relative proportion of seeds sired by self pollen versus those sired by outcross pollen from other conspecific individuals (Barrett & Harder 2017). Flexibility in a plant's mating system allows outcrossing when outcross pollen is not a limiting factor, while providing reproductive assurance through self-pollination when outcross pollen is not readily available (Cheptou 2019, Karron et al. 2012). Shifts in mating systems to higher selfing rates are typically thought to represent a response to low or unpredictable pollination services (Cheptou 2019), but many studies have shown that it can also occur if IPT diminishes the availability of outcross pollen (Bell et al. 2005, Fishman & Wyatt 1999, Randle et al. 2018, Smith & Rauscher 2008). IPT can favor selfing regardless of whether the competing species are closely related or not; for example, one study found that extensive pollen misplacement by foraging bumblebees resulted in

much greater probabilities for stigmas to receive self rather than outcross pollen in Mimulus ringens plants growing in experimental arrays with the distantly-related competitor Lobelia siphilitica (Bell et al. 2005).

In many cases, selfing may occur towards the end of the flower's lifespan as a 'last resort' if little or no outcross pollen was received (Lloyd 1992). However, this does not prevent heterospecific pollen deposition or pollen misplacement from diminishing outcrossing rates, thus such delayed selfing is not expected to be selected for in scenarios where IPT is the main factor influencing the mating system (Goodwillie & Ness 2013, Randle et al. 2018). Preemptive selfing, on the other hand, takes place before the floral bud opens (Lloyd 1992, Sicard & Lenhard 2011), thus securing pollination before any IPT can occur (Randle et al. 2018). Such extreme transitions to a predominantly or fully selfing mating system are also commonly accompanied by a suite of characters termed the "selfing syndrome", including smaller flowers, highly reduced anther-stigma separation distance (herkogamy), lower pollen-to-ovule ratio, diminished pollen production, and limited secretion of nectar and scent (Sicard & Lenhard 2011). Divergence in these floral traits among closely-related species is well documented in several angiosperm taxa (Briscoe Runquist & Moeller 2014, Grossenbacher & Whittall 2011, Kalisz et al. 2012, Vallejo-Marín et al. 2014). In one clear example of selfing in response to IPT, Fishman & Wyatt (1999) found that Arenaria uniflora populations exhibited preemptive selfing, smaller flowers, and lower herkogamy in regions of sympatry with the congener A. glabra, and that outcrossing A. uniflora individuals placed in arrays with A. glabra faced significant decreases in conspecific seed set. A similar study with three Centarium species that exhibit a range of mating systems demonstrated that the earlier that selfing occurs in a flower's lifespan, the more effective it is in reducing costs of IPT from congeners (Brys et al. 2016). For two of these species that overlap greatly in their native and invaded habitats in mainland Europe and the UK (C. erythraea and C. littorale), a separate study found that which of these species evolved decreased herkogamy and increased

selfing depended on which first colonized the site, suggesting that the reproductive assurance value of selfing is higher for late-arriving species as it simultaneously counters the frequency disadvantage and prevents the production of unfit hybrid progeny (Schouppe et al. 2017).

The outcome of IPT interactions between selfers and outcrossers also depends greatly on the differences in their pollen competitive ability in each other's pistils, and these differences almost invariably favors the outcrosser (Brandvain & Haig 2005). Pollen from outcrossing species is well adapted to compete in a wide range of pistil environments whereas pollen from selfers typically fails in outcrossers' flowers. Similarly, stigmas and styles from outcrossing species present much stronger barriers to pollen from selfers than vice versa (collectively termed the "SI x SC rule"; Brandvain & Haig 2005, Goodwillie & Ness 2013, Harder et al. 1993). Thus, species that begin to shift towards selfing due to IPT competition with more outcrossing relatives may face a snowballing selective pressure for such selfing as their pollen lose their competitive ability.

Wide interpopulation variation in mating systems was found to be common across angiosperms in an extensive survey covering 741 populations of 105 species from 80 genera and 44 plant families (Whitehead et al. 2018). This variation could be due to differences across a species' distribution in pollinator environments, IPT interactions with co-flowering plants, or both (Karron et al. 2012). We know of only two cases where researchers attempted to disentangle the importance of these factors. The first involves two recently-diverged subspecies of Clarkia xanthiana (Onagraceae): the outcrosser subsp. xanthiana and the selfer subsp. parviflora. Briscoe Runquist & Moeller (2014) found that 1) pollen limitation was higher and selfing more advantageous in regions where these subspecies co-occurred, 2) the selfer's herkogamy and flower size were significantly reduced in these regions of sympatry, and 3) contrasting pollinator environments did not explain the differences detected between allopatric and sympatric sites (Briscoe Runquist & Moeller 2014). A follow-up study further established that, despite pollen

transfer being reduced due to low flowering overlap and a stronger pollinator preference for the outcrosser, gametic isolation barriers were weaker for the selfer, making it prone to greater costs from maladaptive hybridization with its congener as predicted by the SI x SC rule (Briscoe Runquist et al. 2014). A second striking example found high flowering overlap and pollinator sharing between the sister species Collinsia linearis and C. rattanii in zones of sympatry, but that interspecific movements by pollinators caused highly asymmetric pollen flow from C. linearis to C. rattanii; in line with this observation, C. rattani (and not C. linearis) display significantly earlier preemptive selfing in sympatry (Randle et al. 2018). Although variation in mating systems does not always correlate with co-occurrence patterns among close-relatives (Grossenbacher et al. 2016, Matallana et al. 2010, but see Whitton et al. 2017), there is substantial evidence suggesting that increased selfing rates in sympatry can facilitate coexistence and may be a common evolutionary response to IPT-driven pollen limitation.

4. POLLEN TRANSFER DYNAMICS AND GENE FLOW DURING EARLY DIVERSIFICATION

Although IPT typically causes fitness reductions and selection for floral divergence, its impacts can vary among more closely-related species if it leads to interspecific gene flow. Among interfertile plant species, IPT is in fact the means by which genes are exchanged. We suggest that the classical competition-based view of IPT prevalent in the pollination literature has limited the understanding of its evolutionary importance in angiosperm diversification in terms of speciation and introgression. Hybridization as a consequence of IPT was recognized by Morales & Traveset (2008), but only in the context of gene flow between alien and native species and between genetically-modified crops and their wild relatives. However, rapid advances in our ability to detect and quantify interspecific gene flow using modern genomic and statistical tools (Ellstrand 2014, Payseur & Rieseberg 2016) have revealed widespread evidence of hybridization across

many levels of the Tree of Life. Speciation and reproductive isolation are now known to commonly occur despite ongoing gene flow (Abbott et al. 2013, Baack et al. 2015), and modern tree-thinking has shifted to embrace reticulation (Mallet et al. 2016). Furthermore, evidence suggests that gene flow has contributed significantly to the evolution of many plant clades through adaptive introgression (Ellstrand 2014, Schmickl et al. 2017). Finally, our rapidly changing world is bringing about increasing opportunities for gene exchange via IPT due to range shifts among formerly allopatric plant species (Vallejo-Marín & Hiscock 2016), making it particularly urgent that we study and understand the effects of IPT on patterns of gene movement between species.

Along the continuum of evolutionary divergence, populations, lineages, and species become increasingly differentiated (De Queiroz 2011), and the effects of IPT and resulting gene flow shift with increasing differentiation. Pollen transfer will closely approximate gene flow in early stages of divergence, but they progressively decouple during intermediate and late stages as reproductive isolation increases, until eventually foreign pollen fails to produce any hybrid progeny. Below, we discuss impacts of IPT in three main stages of the divergence continuum.

4.1. Early divergence: homogenizing gene flow and gene flow-selection balance

With little evolutionary divergence and a lack of isolating barriers, IPT should lead to homogenizing gene flow: pollen is transferred, fertilizes ovules, and genes are thus exchanged. The expectation is that the populations will fuse together or form a stable hybrid zone in the point of primary contact (Abbott et al. 2013, Payseur & Rieseberg 2016). Differences in habitat type or pollinator availability outside of the point of contact may favor the formation of a stable hybrid zone due to a balance between selection and gene flow, depending on the rate of IPT and the fitness of hybrids relative to parental populations (Arnold et al. 2008, Campbell et al. 1998).

Absence of such selection outside of the point of contact would make fusion of the two gene pools more likely (Buerkle et al. 2003).

What role do pollinators play in preventing or promoting such fusion of gene pools? Manipulative studies across multiple populations are needed to understand if local adaptation to spatiotemporal variation in pollinator availability and/or IPT dynamics can generate the initial levels of floral and genetic divergence needed to restrict gene flow to some extent. For example, how do fitness costs associated with pollen transfer between populations initially arise and drive incipient reproductive isolation? Do local pollen transfer dynamics and the competitive environment promote local adaptation of pollen/pistil compatibilities that restrict gene flow between populations? Does specialization to different pollinator environments across a plant's range (ecotypes; e.g. Anderson et al. 2010, Newman et al. 2015) result in floral isolation between subpopulations? These questions have only recently begun to be explored by a handful of studies, for example in North American Clarkia with generalized pollination (Briscoe Runquist & Moeller 2014, Briscoe Runquist et al. 2014, Kay et al. 2018, Miller et al. 2014), in bee-pollinated Mimulus (Grossenbacher & Stanton 2014), in South African hawkmoth-pollinated Gladiolus (Anderson et al. 2010), and in long-proboscid-fly-pollinated Nerine (Newman et al. 2015) and Leperousia (Anderson et al. 2016). Unfortunately, comparable multi-site studies are lacking for other biogeographic regions, most notably from the species-rich tropics.

One of these relevant studies, by Kay et al. (2018), examined the role of pollinators in floral isolation between populations of the sister species Clarkia concinna (Onagraceae) and C. breweri via 'experimental sympatry' (Figure 2a). The authors' primary objective was to evaluate whether the shift to hawkmoth pollination by C. breweri conferred floral isolation from the pollinator generalist C. concinna. Common garden experiments revealed remarkable variation in IPT between C. breweri and four different ecotypes of C. concinna (Figure 2b). Specifically, hawkmoths transferred very little pollen from any of the C. concinna ecotypes to C. breweri, nor

from C. breweri to three of the C. concinna, ecotypes, yet transferred strikingly large amounts from C. breweri to the coastal ecotype of C. concinna (Kay et al. 2018; Figure 2b). Thus, this coastal C. concinna would be very likely to incur in hawkmoth-mediated asymmetric IPT interactions (and possibly fitness costs) with C. breweri if they co-occurred together. Notably, another C. concinna ecotype ("South" in Figure 2a) parapatric with C. breweri shows all of the traits typical of the "selfing syndrome" described in Section 3.3, suggesting a shift to selfing was favored by IPT interactions with C. breweri (Kay et al. 2018).

What does this study tell us about early divergence and how initial reproductive isolation might arise? Results demonstrate that floral isolation remains incomplete between C. breweri and C. concinna in either direction, that potential IPT would be mostly asymmetric (C. breweri → C. concinna), and that the various populations of C. concinna are not all equally isolated from their congener. This and similar studies (Grossenbacher & Stanton 2014, Newman et al. 2015) show that selection to local pollinator environments across a species' range might confer ecotypes with different degrees of susceptibility to IPT with close relatives and even with other intraspecific ecotypes. Over enough time, the selective effects of local pollinator environments and local competition via IPT likely often lead to floral divergence and associated reproductive isolation, which would then restrict gene flow among subpopulations and potentially lead to speciation.

One intriguing hypothesis is that even in the absence of differences in habitat, pollinators, or competitors across a species' geographic range, strong sexual selection alone may drive intraspecific divergence and thus ultimately promote speciation. Specifically, outcrossing species constantly face intraspecific competition between males when pollen from multiple males are deposited on stigmas, such that males with pollen that germinates and reaches ovules faster will enjoy higher levels of paternity. At the same time, females may benefit from 'leveling the playing field' between competing males to maximize the diversity of sires among their offspring. Such sexual conflict can lead to local adaptation of compatibility between pollen and stigmas/styles,

thus potentially promoting reproductive isolation among the various subpopulations within a species (Ortiz-Barrientos et al. 2009). Unfortunately, to our knowledge this hypothesis remains untested.

4.2. Intermediate divergence: reinforcement and adaptive introgression

At an intermediate stage of divergence, gene flow will be restricted to some extent but IPT dynamics among two species will still affect patterns of gene flow between them (Campbell et al. 1998, Natalis & Wesselingh 2012b, Surget-Groba & Kay 2013, Zhang et al. 2016). Even if gametic or postzygotic isolation serves to limit gene flow, or constrain it to small parts of the genome (Payseur & Rieseberg 2016), IPT will still ultimately determine whether gene flow occurs. Evolutionarily speaking, it is during this stage when IPT-driven gene flow might have the most profound impacts for plant evolution (Ellstrand 2014), leading to the merging of gene pools, the reinforcement of barriers separating them, and/or adaptive introgression between the species.

If hybrids formed by IPT exhibit particularly low fitness relative to parental species, reinforcing selection may favor strengthening of prezygotic barriers to gene flow (Hopkins 2013, Ortiz-Barrientos et al. 2009). This can include the same adaptations outlined in Section 3: temporal isolation via reduced flowering overlap (Martin & Willis 2007, Zhang et al. 2016), floral isolation via the attraction of different pollinators (Hopkins & Rausher 2012) or differential pollen placement (Kay & Schemske 2008), gametic isolation via increased pollen-pistil incompatibilites (Arceo-Gómez et al. 2016b), or transitions towards self-pollination (Rausher 2017, Schouppe et al. 2017). Such reinforcement of reproductive barriers will ultimately determine the evolutionary course of hybridization and the resulting pattern of gene exchange between the interacting species.

As mentioned previously, there is no reason to believe that any of these various isolating mechanisms should evolve at the same rate between pairs of species, thus we might often expect barriers and associated gene flow between pairs to be asymmetric. In fact, previous assessments of reproductive isolation among angiosperms have found asymmetry to be the norm (Lowry et al. 2008, Tiffin et al. 2001). A survey of 19 species pairs found that prezygotic barriers were on average twice as strong as postzygotic ones, but that the latter were almost three times more asymmetric (Lowry et al. 2008). Among the prezygotic barriers evaluated, pollinator-mediated isolation (= floral isolation) showed the greatest asymmetry: almost twice as high as the other prezygotic barriers, and roughly half as high as the postzygotic ones (Lowry et al. 2008). Regrettably, there have been no quantitative assessments of the extent to which asymmetry in barrier strength correlates with gene flow among diverging species. As a preliminary assessment of this relationship, in Table 1 we review 10 instances of species pairs where there is 1) clear evidence for IPT between the pair, via pollinator sharing, interspecific pollinator movements, and/or transfer of pollen or analogues, 2) sufficient data to quantify asymmetry in the strength of pre-pollination and gametic isolation (following Sobel & Chen 2014), and 3) additional data on gene flow between the pair. To summarize, for four species pairs (Helianthus, Iris fulva¬-I. brevicaulis, Mimulus, and Phlox) pre-pollination and gametic barriers were asymmetric in the same direction, and correctly predicted the direction of introgression. For three others (Ipomopsis, Iris fulva-I. hexagona, and Silene), only gametic isolation was asymmetric, and again correctly predicted the direction of introgression. In one pair (Costus), pre-pollination and gametic barriers were asymmetric in the same direction, but gene flow was symmetric. This mismatch may be due to fertile F1 hybrids crossing equally well with either parental species, nullifying the asymmetry found in pure parental crosses (Surget-Groba & Kay 2013). In the final two pairs (Clarkia and Rhinanthus), gene flow actually followed a pattern opposite to the isolating barriers. Evidence suggests a similar explanation for this mismatch in the case of Rhinanthus, in that backcrossing via fertile hybrids is asymmetrical in the opposite direction (Natalis & Wesselingh 2012b). Thus,

despite some exceptions due to backcrossing, overall the direction of asymmetry in pre-pollination isolation (which equals asymmetry in IPT) and in gametic isolation between pairs of species tends to predict the direction of gene flow, with gametic barriers typically more closely related to gene flow patterns.

In this stage of intermediate divergence between species, IPT-mediated gene flow can also play a profound role in plant evolution by increasing genetic variation and/or by allowing exchange of adaptive traits across species boundaries (Abbott et al. 2013, Schmickl et al. 2017). Such adaptive introgression has been shown for traits related to drought tolerance (Campbell & Waser 2007, Whitney et al. 2010) and floral color (Stankowski & Streisfeld 2015). In some extreme cases, repeated hybridization and backcrossing can lead to the formation of new species reproductively isolated from its parental relatives (Clay et al. 2012, Renaut et al. 2014, Vallejo-Marín et al. 2016).

Despite the large amount of research devoted to the evolution of reproductive isolation and how it restricts gene flow during divergence, many questions remain unanswered. For example, the relationship between IPT and gene flow is expected to be positive during early divergence as more pollen flow leads to more genes exchanged, but how does the relationship change as different isolation processes are reinforced at the pre- and post-pollination stages? Do more highly asymmetric IPT dynamics tend to increase or decrease the chances of reinforcement or the speed of evolution of isolating barriers? And does the degree of asymmetry in IPT between a pair of species tend to decrease over time, as the species facing greater IPT evolves stronger pre-pollination barriers? Finally, the relative contribution of post-pollination (i.e. gametic and postzygotic isolation) versus pre-pollination barriers to total reproductive isolation is expected to increase with increasing evolutionary divergence (Christie & Strauss 2018, Kostyun & Moyle 2017); how do IPT dynamics and resulting gene flow change across these stages of speciation?

We argue that the relationship between IPT and gene flow during speciation represents an exciting and underexplored topic in need of further research.

4.3 Late divergence: reproductive character displacement

Finally, the third stage represents IPT between pairs of species that are already completely reproductively isolated via gametic and/or postzygotic barriers. In these cases, competition through IPT will still negatively impact floral fitness via reproductive interference, by wasting gametes and resources for the plants and decreasing seed set (Morales & Traveset 2008). These costs will select for reproductive character displacement that shifts barriers to earlier-acting stages of reproductive isolation. In other words, if only postzygotic barriers are present, gametic isolation will be favored (to prevent styles from being clogged and ovules from being usurped), and if only post-pollination barriers are present, pre-pollination barriers will be favored to increase temporal isolation, floral isolation (ethological or mechanical), or selfing rates (as described previously in Section 3).

5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Although much research has focused on elucidating the effects of competition for pollination in plant ecology and evolution, a common outcome of this competition, IPT, has received little attention until relatively recent. Our understanding of these competitive interactions will only improve as more research is devoted to the fitness consequences of heterospecific pollen deposition and pollen misplacement in natural plant populations under diverse ecological and evolutionary contexts. In particular, the extent to which IPT affects plant reproduction must be evaluated on multiple pollinator community contexts across species' ranges, over a breadth of phylogenetic distances, and at different spatial scales and habitat configurations. Experimental

manipulations must also be employed whenever feasible to improve our mechanistic understanding of factors influencing IPT dynamics and their outcomes.

The role of IPT and the extent to which it matches gene flow during early plant diversification also warrants more attention. Genomic tools, analytical approaches, and species-level phylogenies readily available for several plant groups constitute valuable resources to investigate the influence of IPT on reproductive isolation and floral evolution. Patterns of recent and ongoing gene flow mediated by IPT and its effects can inform our knowledge about the evolution of reproductive isolation and the maintenance of species boundaries, patterns of adaptive introgression, the rise of floral phenotypic novelty, and shifts in mating systems. One particularly informative approach to examine early divergence involves using experimental sympatry (sensu Kay et al. 2018) to examine the importance of various pre- and post-pollination barriers in preventing gene flow should allopatric subpopulations or incipient species come into secondary contact.

We also need to expand the breadth of plant-pollinator systems studied, since most research involves bee- and bird-pollinated systems in temperate zones. Large-sized pollinators with hairy body surfaces and high vagility such as hawkmoths and bats often carry large pollen loads from multiple plant species (Johnson & Raguso 2016, Muchhala & Jarrín-V 2002), but the extent to which they drive IPT interactions has only been explored by a few studies (Ippolito et al. 2004, Muchhala & Potts 2007, Muchhala & Thomson 2012, Muchhala et al. 2009). Small-bodied bees and flies are similarly understudied, as are tropical plants in terms of studies of competition for pollination generally and IPT interactions more specifically (but see Feinsinger & Tiebout III 1991, Muchhala 2008, Muchhala & Thomson 2012, Muchhala et al. 2014). The only exhaustive and complementary set of studies on pollination, reproductive isolation, gene flow, and speciation among closely-related tropical plants were conducted in the Neotropical spiral ginger genus Costus (Kay 2006, Kay & Schemske 2008, Surget-Groba & Kay 2013).

Finally, more attention to the magnitude and importance of IPT in natural communities will greatly improve our understanding of plant species coexistence and community assembly. This in turn can inform both pure and applied aspects of pollination biology (Mitchell et al. 2009), especially with regard to human-modified environments and plant invasion scenarios, where novel evolutionary interactions between plants and pollinators are taking place (Albrecht et al. 2016, Johnson & Ashman 2019, Vallejo-Marín & Hiscock 2016). Further ecological and evolutionary research on IPT dynamics is necessary to better understand plant-pollination interactions in our rapidly changing world, and will have profound implications for biodiversity conservation and the provisioning of ecosystem services enjoyed by human societies.

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FIGURES

Figure 1

Example of interspecific pollen transfer interactions amongst three sympatric bat-pollinated flowers that exhibit distinct but overlapping pollen placement patterns on their shared bat pollinators (e.g. *Anoura geoffroyi*, Phyllostomidae). Panel *a* shows the pollen placement location for each species indicated by dashed lines and colors: *Centropogon nigricans* (Campanulaceae; green), *Aphelandra acanthus* (Acanthaceae; yellow), and *Burmeistera sodiroana* (Campanulaceae; red). Panel *b* shows the number of pollen grains (± SE) from focal species *A. acanthus* that were transferred by bats to conspecific stigmas following four treatments: without any intervening visit, after an intervening visit to a plastic straw (control), to a female *B. sodiroana* flower, or to a male *B. sodiroana* flower with pollen. Panel *c* shows the results of the experiments where the competitor was *C. nigricans*. Together, both sets of experiments show that greater overlap in pollen placement promotes higher rates of pollen misplacement during alternating visits, therefore increasing the male fitness costs of the competition. In addition, intervening visits to male flowers caused the bats to deposit large amounts of foreign pollen in *A. acanthus* stigmas (95.4 grains from *B. sodiroana* and 115.7 grains from *C. nigricans*, on average; Muchhala & Thomson 2012). Such heterospecific pollen deposition would further impact fitness through the female floral function. Figure adapted with permission from Muchhala & Thomson (2012).

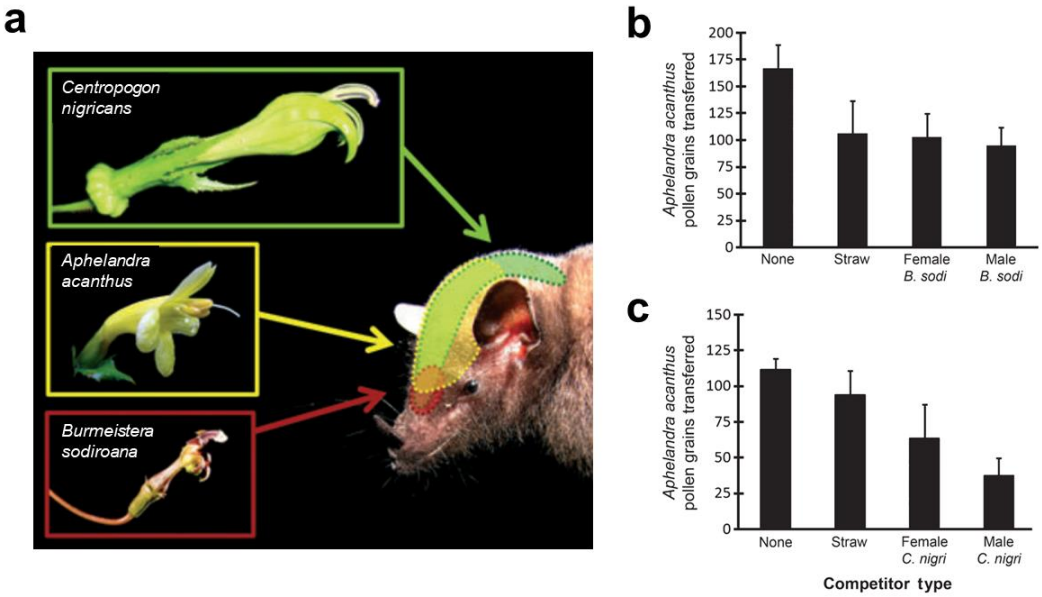
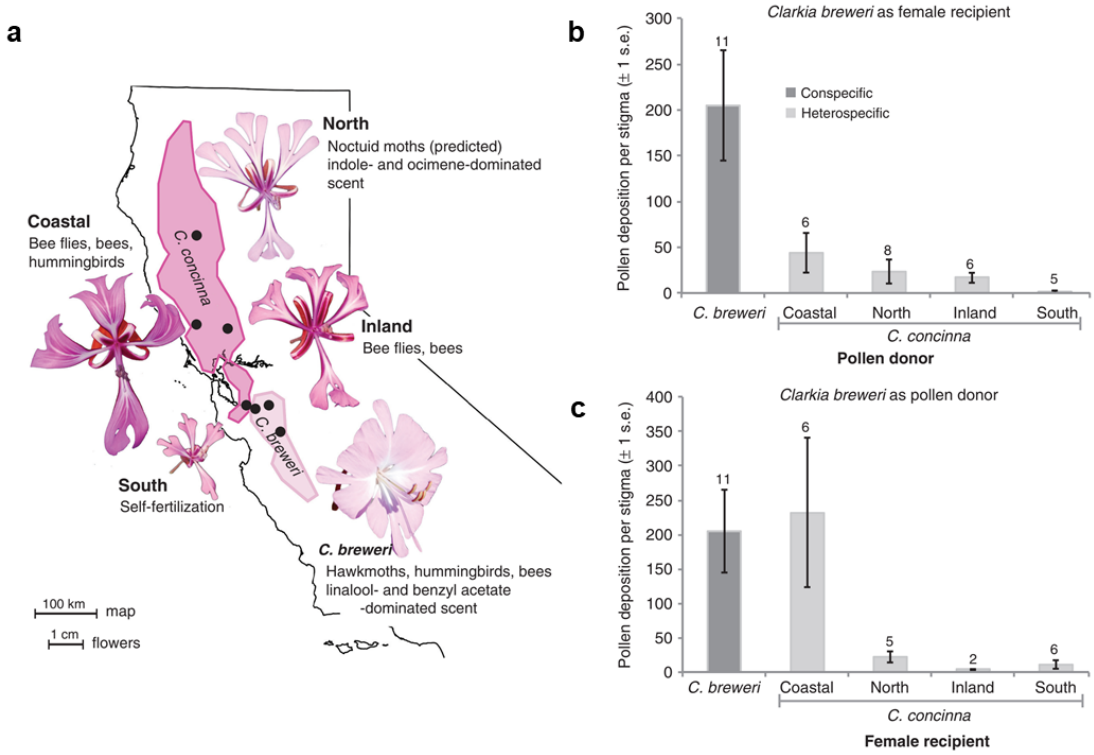

a
Centropogon nigricans
Aphelandra acanthus
Burmeistera sodiroana
b
Aphelandra acanthus pollen grains transferred
200
175
150
125
100
75
50
25
0
None
Straw
Female B. sodi
Male B. sodi
c
Aphelandra acanthus pollen grains transferred
150
125
100
75
50
25
0
None
Straw
Female C. nigri
Male C. nigri
Competitor type

Figure 2

Pollen transfer interactions and floral isolation in experimental sympatry for two recently diverged *Clarkia* species (Onagraceae) from California. Panel *a* shows the geographic occurrence, floral morphology, and pollinators of focal *C. breweri* and four ecotypes of its close relative *C. concinna*. Panels *b* and *c* show pollen deposition per stigma for different floral arrays with *C. breweri* as the female recipient or pollen donor, respectively, alongside the four floral ecotypes of *C. concinna*. Numbers above bars represent the number of experimental arrays including each floral ecotype. Figure adapted with permission from Kay et al. (2019).


a
North
Noctuid moths (predicted)
indole- and ocimene-dominated
scent
Coastal
Bee flies, bees,
hummingbirds
C. concinna
Inland
Bee flies, bees
C. breweri
South
Self-fertilization
C. breweri
Hawkmoths, hummingbirds, bees
linalool- and benzyl acetate
-dominated scent
100 km
map
1 cm
flowers
b
Clarkia breweri as female recipient
Pollen deposition per stigma (± 1 s.e.)
Conspecific
Heterospecific
11
6
8
6
5
C. breweri
Coastal
North
Inland
South
C. concinna
Pollen donor
c
Clarkia breweri as pollen donor
Pollen deposition per stigma (± 1 s.e.)
11
6
5
2
6
C. breweri
Coastal
North
Inland
South
C. concinna
Female recipient

TABLES

Table 1

Asymmetries in pre- and post-pollination isolation and gene flow among 10 diverging species pairs

Focal Group (RI references)	Diverging taxa	Pre-pollination isolation[a]				Gametic isolation[f]				Introgression[i] (gene flow references)
		Barriers[b]	RI[c]	Asymmetry[d]	IPT direction[e]	Barriers[g]	RI[c]	Asymmetry[d]	Crossing direction[h]	
*Clarkia xanthiana subspecies (*Runquist et al. 2014)	*C. xanthiana* subsp. *parviflora*	FO, SP, IMP	0.991	0.045	S, very low	PTG, PC, HSS	0.528	0.411	A (*Cxx→Cxp)*	A (*Cxp→Cxx)* (BC) (Pettengill & Moeller 2012)
	C. xanthiana subsp. *xanthiana*		0.946	-0.045			0.939	-0.411		
Costus (Kay 2006)	*pulverulentus*	SP, PT	1.000	0.820	U (*Cp→Cs)*	PG, PTG, HSS	0.954	0.298	A (*Cp→Cs)*	S (BC) (Surget-Groba & Kay 2013)
	scaber		0.180	-0.820			0.656	-0.298		
Helianthus petiolaris "ecotypes" (Ostevik et 2016)	dune	SP	0.550	0.190	A (dune→non dune)	PC	0.380	0.260	A (dune→non dune)	A (dune→non dune) (Andrew et al. 2012, 2013)
	non dune		0.360	-0.190			0.120	-0.260		
Ipomopsis; (Aldridge & Campbell (2006, 2007), Campbell & Waser (2007))	*aggregata*	IMP	0.578	0.027	S	PC	0.322	0.574	A (*Ia→It*)	A (*Ia→It*) (Wu & Campbell 2005)
	tenuituba		0.551	-0.027			-0.252	-0.574		
Iris; (Arnold et al. 1993, Carney et al. 1994, Burke et al. 1998, Emms & Arnold 2000)	*brevicaulis*	IMP	-0.333	0.698	A (*If→Ib*)	PC	-0.395	0.928	A (*If→Ib*)	A (*If→Ib*) (Arnold et al. 2010)
	fulva		0.365	0.698			0.534	-0.928		

Iris; (Arnold et al. 1993, Carney et al. 1994, Burke et al. 1998, Emms & Arnold 2000)	*fulva*	IMP	0.264	0.000	S	PC, HSS	1.000	0.560	A (*If→Ih*)	A (*If→Ih*) (Arnold et al. (2010))
	hexagona		0.264	0.000			0.440	-0.560		
Mimulus aurantiacus "ecotypes"; (Sobel & Streisfeld 2014)	red	IMP	0.873	0.226	A (red→yel)	PC, HSS	0.087	0.150	A (red→yel)	A (red→yel) (Sobel & Streisfeld 2014)
	yellow		0.647	-0.226			-0.063	-0.150		
Phlox; (Ruane & Donohue 2008, Hopkins & Rauscher 2012)	*cuspidata*	IMP	0.160	0.320	A (*Pc→Pd*)	PC, HSS	0.743	0.350	A (*Pc→Pd*)	A (*Pc→Pd*) (Roda et al. 2017)
	drummondii		-0.160	-0.320			0.393	-0.350		
Rhinanthus; (Natalis & Wesselingh *2012a, 2012b, 2013)*	*angustifolius*	SP, IMP, PT	0.449	0.805	A (*Ra→Rm*), very high	PTG, PC, HSS	0.408	0.006	A (*Ra→Rm*)	A (*Rm→Ra)* (BC) (Ducarme et al. (2010), Vrancken et al. (2012))
	minor		-0.356	-0.805			0.402	-0.006		
Silene; Karrenberg et al. (2018)	*dioica*	FO, PT	0.584	0.066	S, high	PC, HSS	0.247	0.243	A (*Sl→Sd*)	S range-wide; A (*Sl→Sd)* cpDNA in HZ (Minder et al. 2007, Muir et al. 2012)
	latifolia		0.650	-0.066			0.490	-0.243		

[a]Here defined pre-pollination barriers that reduce interspecific pollen transfer, estimated from sympatric populations or by using experimental arrays in sympatry.

[b]FP: flowering overlap; PS: shared pollinators; IMP: interspecies movements by pollinators; PT: direct counts of pollen transferred (or pollen analogue e.g. fluorescent dye).

[c]Cumulative isolation for that reproductive stage estimated following Sobel & Chen (2014). A RI value of 1 equals a gene flow probability of zero (full assortative mating), while a RI value of -1 specifies a gene flow probability of 1 (complete disassortative mating), and a RI value of 0 indicates a gene probability of 0.5 (random mating). Raw data, calculations, and references provided in **Supplemental Table 1**.

[d]Estimated as the absolute value of the difference in isolation for that stage as in Lowry et al. (2008).

[e]Expected prevailing direction of IPT based on the asymmetry of pre-pollination barriers (indicated by an arrow). S: bidirectional symmetric; A: asymmetric; U: unidirectional.

[f]Includes barriers to fertilization and siring success only (post-pollination prezygotic). Postzygotic barriers were also estimated but not included here (see **Supplemental Table 1**).

[g]PG: pollen germination; PTG: pollen tube growth; PC: pollen competition: SSS: seeds siring success

[h]Expected prevailing crossability direction based on the asymmetry of post-pollination barriers (indicated by an arrow). S: bidirectional symmetric; A: asymmetric; U: unidirectional.

[i]Estimated direction of interspecies gene flow found during follow-up studies in the same locations or populations and by the same research group. Direction is shown with the same notation as in e and h. BC: backcrossing. HZ: hybrid zone.

CHAPTER 2.

Pollen transfer dynamics influence the response to heterospecific pollen deposition among co-occurring bat-pollinated *Burmeistera* (Campanulaceae: Lobeliodeae)

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Abstract

Bats are key pollinators of hundreds of tropical plant species but they often carry copious, multispecies pollen loads in their fur. Thus, heterospecific pollen deposition might be common among sympatric bat-pollinated plants which could cause reproductive interference and favor post-pollination isolation. Previous work with sympatric members of the bat-pollinated genus *Burmeistera* found differential tolerance to heterospecific pollen deposition between species that

tend to be donors or recipients of heterospecific pollen. We quantified conspecific and heterospecific pollen deposition for two populations of *Burmeistera ceratocarpa*, a species expected to be recipient in heterospecific pollen transfer interactions, that co-occur with different potential donor relatives (*B. borjensis* and *B. glabrata*). We then used a fully reciprocal cross-pollination scheme using pollen mixtures to test whether the species' responses to heterospecific pollen deposition were related to the patterns of pollen transfer between them at both sites. We did not find differences in conspecific pollen deposition amongst the study species but *B. ceratocarpa* indeed received significantly more heterospecific pollen deposition from its relatives at both sites than viceversa. We also found that increasing amounts of heterospecific pollen in mixtures affected seed production only for *B. borjensis* and *B. glabrata*, but not *B. ceratocarpa*. Thus, heterospecific pollen did not affect conspecific pollination in *Burmeistera ceratocarpa*, suggesting that early acting post-pollination barriers prevent reproductive interference. Allopatric crosses between populations at both sites also revealed that the study species are fully isolated in sympatry, while isolation between allopatric populations is strong yet incomplete. Together, our results support the idea that frequent heterospecific pollen deposition might select for stronger post-pollination barriers to such pollen to alleviate the competitive costs of sharing low fidelity pollinators with co-occurring relatives.

Keywords: pollinator sharing, bat pollination, reproductive interference, floral fitness, seed set, gametic isolation

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When shared pollinators alternate foraging visits between co-flowering plants, pollen might be transferred interspecifically and lead to reproductive interference (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019a). Heterospecific pollen arriving on a stigma can affect reproduction by preventing successful adhesion and germination of conspecific pollen grains, and if the species are closely-related enough heterospecific pollen might be able to produce pollen tubes that compete or interfere with conspecific pollen tubes in the style (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013; and references therein). Such reproductive interference can have profound evolutionary consequences when it occurs between sympatric relatives. If the interacting species are interfertile, heterospecific pollen deposition will lead to hybridization which would be maladaptive unless hybrids' fitness equals or surpasses that of the parentals. However, if the species are already fully reproductively isolated heterospecific pollen deposition will carry out negative fitness costs by diminishing opportunities for successful conspecific pollination and seed production. In either of these two scenarios, it is expected that frequent heterospecific pollen deposition will be detrimental for a species and thus favor the evolution of pre- and post-pollination isolation barriers (Moreira-Hernández and Muchhala, 2019). The role of pre-pollination (*i.e* pollinator) isolation in preventing reproductive interference has received considerable attention in the literature (Muchhala and Potts, 2007; Huang and Shi, 2013; Armbruster et al., 2014; Whitehead and Peakall, 2014; Kay et al., 2019), but less attention has been given to how post-pollination isolation barriers respond to natural rates of heterospecific pollen deposition.

Among bat-pollinated plants, many studies have raised the possibility that heterospecific pollen deposition by bats might be common given their relatively large size and densely-furred bodies that commonly carry copious, multispecies pollen loads (Muchhala and Jarrín-V, 2002; Muchhala et al., 2009; Stewart and Dudash, 2016). Not surprisingly, many bat-pollinated species

have evolved specialized flowers with elaborate flower morphologies to avoid reproductive interference by depositing pollen on different areas of the bats' bodies (*i.e.* differential pollen placement; Tschapka et al., 2006; Muchhala, 2008; Stewart and Dudash, 2017). However, it is less clear the extent to which bat-pollination plants have adapted post-pollination barriers to reduce effects after heterospecific pollen has arrived to stigmas. Our past study with a sympatric species pair of bat-pollinated *Burmeistera* bellflowers (Campanulaceae) found differential effects of heterospecific pollen deposition on reproduction between the two focal species. Using mixtures containing varying degrees of conspecific and heterospecific pollen, we found that *B. ceratocarpa* was still able to successfully produce many seeds under increasing amounts of heterospecific pollen from its congener *B. borjensis*, while the latter suffered a significant decrease in seed production with increasing amounts of pollen from *B. ceratocarpa* (Moreira-Hernández et al., 2019). Differences in the exsertion of the floral reproductive parts (i.e. exsertion length; Muchhala, 2006) and field experiments suggest that in natural conditions bats transfer pollen predominantly from the long-exserted *B. borjensis* to the short-exserted *B. ceratocarpa* but very little in the opposite direction (Muchhala and Potts, 2007; Muchhala, 2008). Thus, we posited that frequent heterospecific pollen deposition from *B. borjensis* in sympatry might had favored strong post-pollination isolating barriers conferring tolerance against negative effects on reproduction in *B. ceratocarpa* (Moreira-Hernández et al., 2019). Testing this idea is the goal of the study presented here.

In this study we expand upon our previous work (Moreira-Hernández et al., 2019) and investigate whether patterns of pollen transfer between sympatric *Burmeistera* species could potentially explain differences in their response to heterospecific pollen deposition from each other. Specifically, our past study showed that the short-exserted *B. ceratocarpa* had a high tolerance to heterospecific pollen deposition from its relative long-exserted *B. borjensis* (Moreira-Hernández et al., 2019), and we hypothesized that this could be because in natural conditions bat

pollinators transfer pollen from the latter to the former more frequently than viceversa thereby favoring the evolution of strong post-pollination barriers in *B. ceratocarpa*. In this study we quantified rates of pollen transfer and added more detailed hand-pollination experiments to gain more information on crossing patterns and reproductive interference between these species. First, we measured nightly deposition of heterospecific and conspecific pollen to test whether there is in fact asymmetric pollen transfer between the study species. Second, we performed a set of conspecific pollinations as controls to compare fruit and seed production against our mixed pollinations. Third, we also conducted fully heterospecific crosses between both species to confirm whether the study species were able to set heterospecific seeds. Fourth, we also repeated experiments in a second site were *B. ceratocarpa* co-occurs with a different long-exserted species, predicting similar patterns of pollen transfer and post-pollination barrier strength for this species in both sites. Conversely, we also predicted that the two long-exserted species would receive little pollen from *B. ceratocarpa* in either site, and have not evolved strong post-pollination barriers to reduce reproductive interference. Finally, we performed heterospecific crosses between allopatric populations of the study species from both sites. We expected that any post-pollination barriers will evolve in response to the locally co-occurring species, thus should not affect the success of heterospecific crosses between allopatric populations.

MATERIALS AND METHODS

Focal taxa and study sites. — The Neotropical genus *Burmeistera* H. Karst. & Triana (Campanulaceae: Lobelioideae) comprises ~130 species of terrestrial and hemi-epiphytic herbs and shrubs found in cloud forests at middle and high elevations from Guatemala to Northern Peru (Lammers, 2007; Knox et al., 2008; Lagomarsino et al., 2014). The highest diversity of the genus is found in Colombia (~80 spp) and Ecuador (~50 spp), where cloud forest locations typically harbor one to four (but sometimes up to eight) sympatric *Burmeistera* species (Lammers, 2007;

Mashburn, 2019). Flowering overlap between species is extensive as individual plants produce flowers over several months and population level flowering occurs year-round (Muchhala, 2006). Flowers are zygomorphic (bilaterally symmetrical) and protandrous, with reproductive parts exserted outside of the corolla tube opening by a staminal column (Muchhala, 2006, 2008; Figure 1). At anthesis, the corolla tube opens and anthers release copious pollen from the tip of the staminal column initiating the male phase which lasts 24-48 h. The transition to female-phase begins when the stigma protrudes from inside of the staminal column expanding outwards and pushing off any remaining pollen (thus preventing self-pollination; Muchhala, 2006). During the female-phase the stigma surface changes from wet, bright, and smooth for the first couple of days to dry, dull, and withered before flowers are eventually shed. The majority of *Burmeistera* species are pollinated primarily by bats, with hummingbird pollination being restricted to only a handful of species (Muchhala, 2006; Lagomarsino et al., 2017). Fruits in the genus are either fleshy or inflated hollow berries which contain thousands of small seeds (Lagomarsino et al., 2014; Gamba et al., 2017).

Fieldwork was carried out in two cloud forest locations in northeast Ecuador. The first, Yanayacu Biological Station (0°36’03” S, 77°53’22” W; hereafter Yanayacu) is a private biological reserve located at ~2100 masl within the Cosanga River valley and close to the small town of Cosanga. The station borders the much larger Antisana Ecological Reserve (1200 km^2) and supports a mosaic of abandoned pastures and second growth with mature cloud forest found in the upper parts of the property along ridgetops. At this site we studied the long-exserted species *B. borjensis* and the short-exserted *B. ceratocarpa* (Figure 1), which are common in the forest understory and occasionally along forest edges. In Yanayacu the exsertion length of *B. borjensis* is 24.5 ± 2.7 mm (N =18) and that of *B. ceratocarpa* is 16.6 ± 0.8 mm (N = 12). The second location, Cordillera de los Guacamayos (0°37’22” S, 77°50’26” W; hereafter Guacamayos), is a forested mountain ridge at approximately 2250 masl found within the Antisana

Ecological Reserve. Although this site is located only ~5 km away from Yanayacu following a straight line, it is found on the Amazon-facing side of the slopes bordering the Cosanga River valley to the east and thus it is much more humid and has a strikingly different forest composition (Moreira-Hernández and Muchhala, personal observation). At Guacamayos, we studied a second *B. ceratocarpa* population and the sympatric *B. glabrata*, which replaces *B. borjensis* as the local long-exserted species. The main accessible trail follows tall mature cloud forest where *B. glabrata* and *B. ceratocarpa* are very common along the trail and on small forest gaps. At this site, *B. glabrata* flowers have an exsertion length of 23.3 ± 1.8 mm (N = 12) whereas that of *B. ceratocarpa* is 15.7 ± 0.5 mm (N = 15). Flowers of all four populations of the three study species are bat-pollinated and are similar for most floral traits other than exsertion length and the size and shape of the calyx lobes (Figure 1).

Estimating conspecific and heterospecific pollen deposition. — We quantified conspecific and heterospecific pollen deposition for the study species at both sites following methods previously used with *Burmeistera* (Muchhala, 2003, 2006). Staminal columns of flowers in the field were wrapped with a thin layer of parafilm and we placed a 0.5 x 0.8 cm rectangle of clear double-sided tape at the tip of the column where the stigma is located. After 24 h, we collected the tape samples, placed them in microscope slides, and covered them with clear single-sided tape. Previous data showed that diurnal pollen deposition by hummingbirds is negligible (Muchhala, 2006), thus, even though the tapes were left for 24 h on the flowers we expect that the pollen samples primarily reflect nightly pollen deposition by bats during the first 8-12 h. Pollen samples were stained with fuchsin dye gelatin cubes and observed under a light microscope to identify and count all pollen found along two perpendicular transects passing through the center of the tape rectangle. For each species pair, the stained pollen grains could be identified to species due to differences in grain size and the shape of the colpii. Pollen counts allowed us to estimate

conspecific pollen deposition for each study species, as well as heterospecific pollen deposition from the other member of the species pair at each of our two study sites.

Reciprocal cross-pollination experiments. — We used a fully reciprocal mixed pollination scheme to study the effect of heterospecific pollen deposition on fruit and seed production in each sympatric *Burmeistera* species pair (*i.e. B. glabrata* and *B. ceratocarpa* in Guacamayos; *B. borjensis* and *B. ceratocarpa* in Yanayacu). We selected 15-20 focal plants from each species at each site choosing individuals with many open flowers and buds for the experiments. Other individuals were also used opportunistically as pollen donors. We made pollen mixtures using four fresh male flowers from the same site, varying the ratio of flowers used from each type to make mixtures approximating different relative amounts of heterospecific and conspecific pollen. For example, a pollen mixture made using one *B. borjensis* flower and three *B. ceratocarpa* flowers had a 1:3 ratio of heterospecific:conspecific pollen for pollinating *B. ceratocarpa*. Conversely, the same mixture could be used as a 3:1 mixture for pollinating *B. borjensis*. These pollen mixtures were then used in sympatric crosses between the species pair each location. We used four pollen mixture ratios as treatment levels corresponding to increasing heterospecific pollen presence in each mixture: 1:3, 2:2, 3:1, and 4:0 (*i.e.* a pure heterospecific mixture). We also made pure conspecific (0:4) pollen mixtures as controls using four flowers from other conspecific individuals of the same population. Because these pollen ratios are approximations and not actual known quantities, throughout this study we refer to our treatments as ratios of heterospecific to conspecific flowers used in each mixture. Finally, we also performed pure heterospecific pollinations between allopatric populations of the study species to evaluate whether heterospecific pollen from non-co-occurring relatives resulted in successful fruit and seed production. In these allopatric crosses, we pollinated *B. glabrata* and *B. borjensis* using pollen from *B. ceratocarpa* from the population in the opposite location (*i.e.* Yanayacu for *B. glabrata* and Guacamayos for *B. borjensis*). Similarly, for each *B. ceratocarpa* population we

used pollen from the respective long-exserted species that was allopatric (i.e. *B. glabrata* for *B. ceratocarpa* from Yanayacu and *B. borjensis* for *B. ceratocarpa* from Guacamayos).

We replicated each pollination treatment in at least 10 flowers per species at each location. The experiments at Yanayacu for the 1:3, 2:2, and 3:1 mixed pollination treatments were conducted during field seasons in 2014 and 2017 (Moreira-Hernández et al., 2019); the pure conspecific, pure heterospecific, and allopatric crosses in Yanayacu as well as all replicates from Guacamayos were performed between January-March 2019. We applied different treatments within individual plants whenever possible selecting them at random during the course of fieldwork. We were also careful to never use self-pollen in any pollen mixtures applied to a particular stigma. During the first set of experiments in 2014 and 2017 at Yanayacu, treatments were applied to female flowers early in the evening only if visual inspection with a hand lens indicated that pollen had not been deposited on the stigma. Bats' deposit hundreds of pollen grains per visit (Muchhala, 2003) which changes the stigma appearance from shiny to a matte dusty look (Moreira-Hernández & Muchhala personal observation). Thus, after careful examination we assumed that shiny bright stigmas from flowers had just entered female phase and were free of pollen. We did not use any flowers whose stigmas did not have that shiny bright appearance or if they had any pollen grains on them. For all other experiments that we conducted in both locations in 2019, we bagged flowers nearing the end of male phase, precluding the need to visually examine the stigma for previously-deposited pollen.

To apply the pollen mixtures to flowers, we used dry bat skins stuffed with cotton that were prepared following standard procedures for mammal specimens in biological collections (Hall, 1962). We simulated pollen deposition by bats by placing the mixture in the respective area of the bat heads' that would contact each type of flower (i.e. the tip of the snout for *B. ceratocarpa* and the forehead for *B. glabrata* and *B. borjensis*) and then applied it to stigmas early in the evening. We used two different bat specimens for the experiments and every night

each of them was used for only one pollen mixture type combination. Specimens were reloaded with pollen mixtures before every pollination and were thoroughly cleaned off pollen with clear tape at the end of the evening. We believe that this method of pollen application reflects the large amount of pollen bats carry on their fur and deposit in natural conditions (Muchhala, 2003; Muchhala and Thomson, 2010). Following each pollination, we covered the flowers to prevent any further pollen deposition by floral visitors. We then marked and labeled the flower pedicel and the subjacent branch node with tape. We revisited the plants after five weeks to ascertain fruit fate (matured, aborted, or lost), and mature fruits were collected in 70% alcohol and transported to the lab to estimate total seed production per fruit.

Statistical analyses. — To test for differences in conspecific and heterospecific pollen deposition between the study species and to determine the effect of increasing heterospecific pollen deposition on fruit and seed production, we used generalized linear mixed models (GLMM) implemented in the package *glmmTMB* (Brooks et al., 2017) using the *R* statistical software (R Development Core Team 2021). For each species pair at each site, we modelled pollen deposition per flower over a 24 h period using a negative binomial distribution with species and pollen deposition type (conspecific or heterospecific) as fixed factors. We also build a binomial GLMM to determine the effect of heterospecific pollen deposition on fruit abortion rates for each species pair at each site, using species and pollination treatment as fixed effects in the model. Finally, we tested for the effect of heterospecific pollen deposition on seed production by the study species with a negative binomial GLMM specifying species and pollination treatment as fixed factor. In all models, the identity of the plant bearing each flower was also included as a random factor. When pollination treatment effects were significant, we tested for variation across levels using the Tukey-Bonferroni *P* value adjustment for multiple comparisons using the R package *multcomp* (Hothorn et al., 2008).

RESULTS

Patterns of heterospecific pollen deposition among the study species. — Quantification of pollen deposition samples revealed distinct patterns of conspecific and heterospecific pollen receipt among the study species (Figure 2). The species pair at each location showed similar nightly deposition of conspecific pollen grains but experienced different amounts of heterospecific pollen deposition. In Guacamayos, the number of conspecific pollen grains deposited on stigmas for *B. glabrata* and *B. ceratocarpa* was not significantly different (mean ± SD: *B. glabrata*: 109.54 ± 47.27, $N = 46$; *B. ceratocarpa*: 80.47 ± 39.16, $N = 45$; Likelihood ratio test: $X^2 = 1.97$, $P = 0.2413$; Figure 2). On the other hand, heterospecific pollen deposition differed between the species as *B. ceratocarpa* experienced substantial pollen deposition from *B. glabrata* (mean ± SD: 41.42 ± 29.64, $N = 45$) while the latter received very little pollen from the former (mean ± SD: 1.73 ± 3.76, $N = 46$; Likelihood ratio test: $X^2 = 37.84$, $P < 0.0001$; Figure 2). We also found a significant difference in terms of frequency; only 29.9 % of *B. glabrata* samples had some *B. ceratocarpa* pollen while pollen from the former was found in 91.1 % of the samples from the latter (Chi-square test: $X^2 = 11.93$, df = 1, $P = 0.0005$).

At Yanayacu, conspecific pollen deposition was slightly but significantly higher for *B. borjensis* than for *B. ceratocarp*a (mean ± SD: *B. borjensis*: 74.33 ± 40.91, $N = 63$; *B. ceratocarpa*: 45.17 ± 31.83, $N = 63$; Table 2; Figure 2). However, *B. borjensis* received very few *B. ceratocarpa* pollen grains (3.17 ± 6.47, $N = 63$) while *B. ceratocarpa* received a low but significant number of *B. borjensis* pollen grains (12.51 ± 15.11, $N = 63$; Figure 2). Frequency of heterospecific pollen deposition also differed between both species, with 23.8 % of *B. borjensis* samples and 57.1 % of *B. ceratocarpa* samples having some pollen from their respective congener (Chi-square test: $X^2 = 8.82$, df = 1, $P = 0.0030$).

Effects of heterospecific pollen deposition on female reproduction. — We pollinated 333 flowers of both species pairs with at least 10 repetitions per pollination treatment (Table 1). In

Guacamayos, we pollinated 99 flowers of *B. glabrata* and 69 of *B. ceratocarpa* across all treatments. *Burmeistera glabrata* flowers pollinated with pure conspecific pollen had the lowest abortion rates (20%) while these were comparable among flowers pollinated with pollen mixtures (40-53%; Table 1). In contrast, abortion rates by *B. ceratocarpa* were similar among the conspecific control flowers and those pollinated using pollen mixtures (27-40%; Table 1). In both species, all fruits resulting from pure heterospecific pollinations were aborted (Table 1). Contrary to expectations, however, analysis of fruit abortion rates showed that pollination treatment did not affect abortion rates by *B. glabatra* and *B. ceratocarpa* in Guacamayos as neither this factor nor its interaction with the species term were significant (pollination treatment: $X^2 = 5.991$, $P = 0.1120$; species: $X^2 = 0.271$, $P = 0.6026$; pollination treatment *x* species interaction: $X^2 = 1.090$, $P = 0.7796$; Figure 3A).

In Yanayacu, we pollinated 98 flowers of *B. borjensis* and 67 flowers of *B. ceratocarpa*. Flowers of *B. borjensis* aborted fruits at similar rates in the conspecific pollen treatment and in those using pollen mixtures (38-50%; Table 1). Abortion rates also showed low variation among *B. ceratocarpa* flowers from the conspecific pollen treatment and the mixed pollinations treatments (10-28%; Table 1). As with the previous species pair, all heterospecific pollinations in both species resulted in fruit abortion (Table 1). Our analyses showed that fruit abortion rates were significantly lower for *B. ceratocarpa* than for *B. borjensis* (species: $X^2 = 6.925$, $P = 0.0085$; Figure 3B). However, pollination treatment had no effect on abortion rates in either *B. borjensis* or *B. ceratocarpa* (pollination treatment: $X^2 = 0.976$, $P = 0.8070$; pollination treatment *x* species interaction: $X^2 = 1.483$, $P = 0.686$; Figure 3B).

Our analyses showed that pollination treatment had an overall significant effect on seed production (Table 2; Figure 4). Flowers pollinated using mixtures with greater amounts of heterospecific pollen resulted in fruits with fewer seeds (Figure 4). However, the species term and its interaction with pollination treatment were also both significant in our mixed effect model

indicating species-specific differences (Table 2). Both *B. glabrata* in Guacamayos and *B. borjensis* in Yanayacu produced significantly fewer seeds in those treatments where pollen mixtures contained high relative amounts of heterospecific pollen from *B. ceratocarpa* (Table 2; Figure 4). Within *B. ceratocarpa*, on the other hand, total number of seeds per fruit was similar across all pollination treatments in both locations regardless of the composition of the pollen mixture that was used (Table 2; Figure 4). Thus, *B. ceratocarpa* seed production was unaffected by the relative amount of heterospecific pollen from either of its congeners in the pollen mixtures that were applied to flowers.

Finally, our allopatric crosses showed that the populations of our study species from both sites are strongly but not completely isolated from each other. Although fruit abortion rates were still very high (>70%), a small number of fruits developed from heterospecific crosses between allopatric populations of the study species (Figure 5; Table 3). As mentioned above, all heterospecific crosses between sympatric species resulted in fruit abortion. However, when long-exserted *B. glabrata* and *B. borjensis* were pollinated with pollen from the *B. ceratocarpa* population from the opposite location, a handful of the crosses formed fruits in both species although with a lower number of seeds than conspecific controls (Figure 5; Table 3). The same occurred in *B.ceratocarpa*. Pollinating *B. ceratocarpa* from Guacamayos with *B. borjensis* pollen from Yanayacu produced fruits in two instances (Figure 5; Table 3). Similarly, two times *B. ceratocarpa* from Yanayacu developed fruits after pollinations with pollen from *B. glabrata* from Guacamayos (Figure 5; Table 3). In both cases the number of seeds produced was also lower than in conspecific controls (Table 3). Even though the number of pollinations and fruits produced were low in all these cases, these results suggest that post-pollination isolation is apparently complete in sympatry but slightly weaker between allopatric populations of the study species.

DISCUSSION

This study demonstrates that sympatric *Burmeistera* species have evolved strong post-pollination isolation to prevent hybridization and shows that these barriers seem to be specific to co-occurring populations. By quantifying patterns of pollen deposition as well as fruit and seed production under different levels of heterospecific pollen deposition, we show that in *Burmeistera* the strength of post-pollination reproductive isolation is asymmetric and stronger in species that frequently receive heterospecific pollen in nature. The short-exserted *B. ceratocarpa* experienced substantial heterospecific pollen deposition from its relatives in both of our study sites yet was able to attain high fruit and seed production in our hand-pollination crosses even at the highest ratios of heterospecific to conspecific pollen (3:1). Thus, this species has evolved efficient post-pollination isolation mechanisms that limit any reproductive interference caused by heterospecific pollen. In contrast, the long-exserted *B. borjensis* and *B. glabrata* both rarely receive foreign pollen in nature, and in our hand pollinations suffered a decrease in seed set at intermediate and high levels of heterospecific pollen deposition. However, it is worth highlighting that none of the heterospecific crosses between co-occurring species resulted in the production of hybrid seeds, thus all three species have complete post-pollination reproductive isolation. The fact that heterospecific crosses between the allopatric populations did often result in hybrid seeds suggests that such reproductive isolation has been selected for in sympatry, and represents species-specific adaptations to the local heterospecific pollen a species is exposed to. Taken together, our results support the hypothesis that in *Burmeistera* the frequent receipt of heterospecific pollen selects for increased post-pollination isolation that limits reproductive interference in sympatry and prevents foreign pollen from affecting conspecific pollination.

Heterospecific pollen deposition by bats. — Our three study species received similar amounts of conspecific pollen per stigma (Figure 2). However, we observed a high frequency and intensity of heterospecific pollen receipt in *B. ceratocarpa* and very little in either of its long-exserted relatives. Heterospecific pollen transfer interactions in the wild are typically asymmetric

and entail greater costs for one of the interacting species (Briscoe-Runquist and Stanton, 2013; Randle et al., 2018; Moreira-Hernández and Muchhala, 2019). In the case of *Burmeistera*, field data and experiments have shown that pollen movement between species occurs primarily from long- to short-exserted species (Muchhala and Potts, 2007; Muchhala, 2008), in line with the pattern we observed amoung our focal species. Thus, short-exserted species such as *B. ceratocarpa* could be under constant exposure to reproductive interference from heterospecific pollen deposition from sympatric long-exserted relatives. Provided this asymmetry is maintained over sufficient evolutionary time, short-exserted species would be under strong selection to develop effective post-pollination barriers to buffer against reproductive interference caused by heterospecific pollen. Another factor which may impact these heterospecific pollen transfer interactions is the population density of the species involved. At both of our sites, *B. borjensis* and *B. glabrata* are much more abundant than *B. ceratocarpa* and thus likely attract more bats to their flowers and deposit more pollen on their bodies. Both floral exsertion and abundance differences could simultaneously cause greater heterospecific pollen transfer towards *B. ceratocarpa*, thus imposing selection on this species to limit reproductive interference.

Our results also shed light on the occurrence of heterospecific pollen deposition by bat pollinators. Sympatric bat-pollinated plants frequently differ in where they place their pollen on bats' bodies (Muchhala and Jarrín-V, 2002; Tschapka et al., 2006; Muchhala, 2008; Muchhala and Thomson, 2012; Stewart and Dudash, 2017), but inherent imprecision in the pollination process probably exposes stigmas of bat-pollinated flowers to frequent deposition of foreign pollen (as seen in this study). Tolerance to heterospecific pollen deposition might be an important factor driving the reproductive success of many bat-pollinated plants that would be easy to overlook. Whether tolerance to heterospecific pollen deposition occurs in other bat-pollinated plants as a mechanism to alleviate costs to reproduction deserves more research.

Heterospecific pollen deposition and fruit and seed production. — Our fully reciprocal cross-pollination design revealed the patterns of post-pollination isolation between our *Burmeistera* study species. None of the species produced fruits in sympatric crosses using pure heterospecific pollen, confirming that they are not hybridizing in sympatry (Figure 3). However, increasing levels of heterospecific pollen deposition revealed species differences that were observed at the stage of seed production. The two populations of short-exserted *B. ceratocarpa* achieved high seed production across increasing ratios of heterospecific to conspecific pollen applied to their stigmas (Figure 4). In contrast, long-exserted *B. borjensis* and *B. glabrata* showed reductions in seed production when high amounts of heterospecific pollen from *B. ceratocarpa* were applied (Figure 4). Finally, allopatric crosses between our study species using pure heterospecific pollen resulted in low fruit and seed production (Figure 5). Together, these results suggest that (1) our study species exhibit complete post-pollination isolation in sympatry, (2) these isolating barriers are more efficient in *B. ceratocarpa* to the point that even high amounts of heterospecific pollen did not noticeably affect conspecific pollination, and (3) post-pollination isolation in these species is weaker between allopatric populations.

Sympatric populations of close relatives are often isolated by post-pollination barriers that limit hybridization. These barriers are often asymmetric, such that the pistil of one species is more successful at arresting pollen germination and pollen tube growth from its congener, than vice versa (Tiffin et al., 2001; Figueroa-Castro and Holtsford, 2009; Natalis and Wesselingh, 2012; Matallana et al., 2016; Moreira-Hernández and Muchhala, 2019). In our sympatric crosses we observed that pure heterospecific pollinations did not lead to fruit and seed production, indicating that post-pollination isolation mechanisms limiting hybridization are at play among our study species. However, though hybridization is being prevented, reproductive interference can still occur if the presence of heterospecific pollen and pollen tubes affect conspecific pollen performance and seed production. This is what we observed in *B. borjensis* and *B. glabrata* after

our mixed pollinations; the deposition of heterospecific pollen was detrimental to seed production in these species even when relatively high amounts of conspecific pollen grains were present. These two species are not often exposed to this type of reproductive interference, however, because they rarely receive heterospecific pollen in nature. In contrast, heterospecific pollen did not seem to interfere with conspecific pollen success in *B. ceratocarpa*, as this species was able to produce many seeds across a range of relative amounts of heterospecific and conspecific pollen in the mixtures that were applied to stigmas. Thus, post-pollination barriers acting in *B. ceratocarpa* pistils help prevent both hybridization and reproductive interference, likely making this species able to tolerate the frequent heterospecific pollen deposition it experiences from its sympatric relatives.

Post-pollination reproductive barriers can occur at various stages between pollen deposition and ovule fertilization. Early-acting barriers operate in the stigma or the distal part of the style arresting pollen germination and early pollen tube growth, whereas late-acting barriers occur further towards the base of the style and the entrance to ovules preventing fertilization (Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019). Thus, early acting barriers are more effective at limiting reproductive interference, because as heterospecific pollen germinates and grows tubes down the style the opportunities for it to negatively affect conspecific pollen success increase (Ashman and Arceo-Gómez, 2013). For example, stigmas of *B. ceratocarpa* might have been able to arrest foreign pollen germination early on and thus allow conspecific pollen to grow tubes down the style unobstructed by heterospecific pollen tubes. This would be consistent with our observation that seed production did not vary across pollination treatments in *B. ceratocarpa*, even when the ratios of heterospecific to conspecific pollen in mixtures where roughly equal or even greatly skewed towards the former (*e.g.* 2:2 and 3:1; Figure 4). On the other hand, lack of early-acting barriers in *B. borjensis* and *B. glabrata* could have allowed heterospecific pollen tubes to grow down the style and clog the stylar tissue, interfering

with conspecific pollen tube performance. This also would be consistent with the fact that the reduction in seed production by *B. borjensis* and *B. glabrata* occurred only when intermediate and high relative amounts of heterospecific pollen were applied in mixed pollinations. Thus overall, our results suggest that post-pollination isolation acts early on in *B. ceratocarpa* before foreign pollen can negatively interfere with conspecific pollen and pollen tube growth. This does not seem to be the case in the other two species, which are then vulnerable to reproductive interference following heterospecific pollen deposition.

One particularly intriguing result of our study was that post-pollination isolation was incomplete between allopatric populations of the study species, in that hybrid seeds were occasionally produced, while pure heterospecific crosses between sympatric individuals of the study species failed in all cases (Figure 5). This seems indicative of increased reproductive isolation following secondary contact *i.e.* sympatric populations are expected to evolve stronger isolating barriers than allopatric populations to prevent hybridization (Coyne and Orr, 2004; Kay and Schemske, 2008). An example of this process occurs in the Neotropical genus *Costus*, where a pair of species have evolved strong post-pollination isolation in sympatry but, notably, this barrier was much weaker between allopatric populations (Kay, 2006; Kay and Schemske, 2008). In that study, the authors concluded that the presence of post-pollination isolation strictly in sympatry suggest that avoiding hybridization has been selected for in co-occurring populations (Kay, 2006; Kay and Schemske, 2008). We suspect that a similar process of reinforcement is probably at play in *Burmeistera*, with increased post-pollination isolation being favored in sympatry for all species, and even stronger/earlier acting post-pollination isolation favored in *B. ceratocarpa* given the frequent pollen deposition it experiences from its sympatric relatives.

Conclusion. — This study corroborates the hypothesis that patterns of pollen movement by shared pollinators can be related to how species respond to heterospecific pollen deposition. In *Burmeistera*, bat pollinators transfer pollen between sympatric species asymmetrically causing

some species to receive foreign pollen very frequently while others rarely do so. Constant exposure to pollen from sympatric relatives seems to have facilitated the evolution of strong post-pollination reproductive isolation in this group. For *B. ceratocarpa*, the species that receives the largest amount of foreign pollen, these post-pollination barriers are strong enough to prevent even high amounts of foreign pollen from affecting conspecific pollination success. In contrast, two other *Burmeistera* species that do not commonly receive foreign pollen failed to produce many seeds after mixed pollinations with high relative amounts of heterospecific pollen. Post-pollination barriers in *Burmeistera* thus seem to be asymmetric, but in the opposite direction to pollen transfer between species, with early-acting barriers conferring tolerance to foreign pollen for species that are common recipients. Importantly, we show these barriers are stronger in sympatry where they serve to limit reproductive interference. Although further research will be needed to determine whether our results are applicable across other species of *Burmeistera*, or other bat-pollinated plants, our study shows that frequent heterospecific pollen deposition can favor post-pollination isolation to alleviate the reproductive costs of sharing low fidelity pollinators with sympatric relatives.

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FIGURES

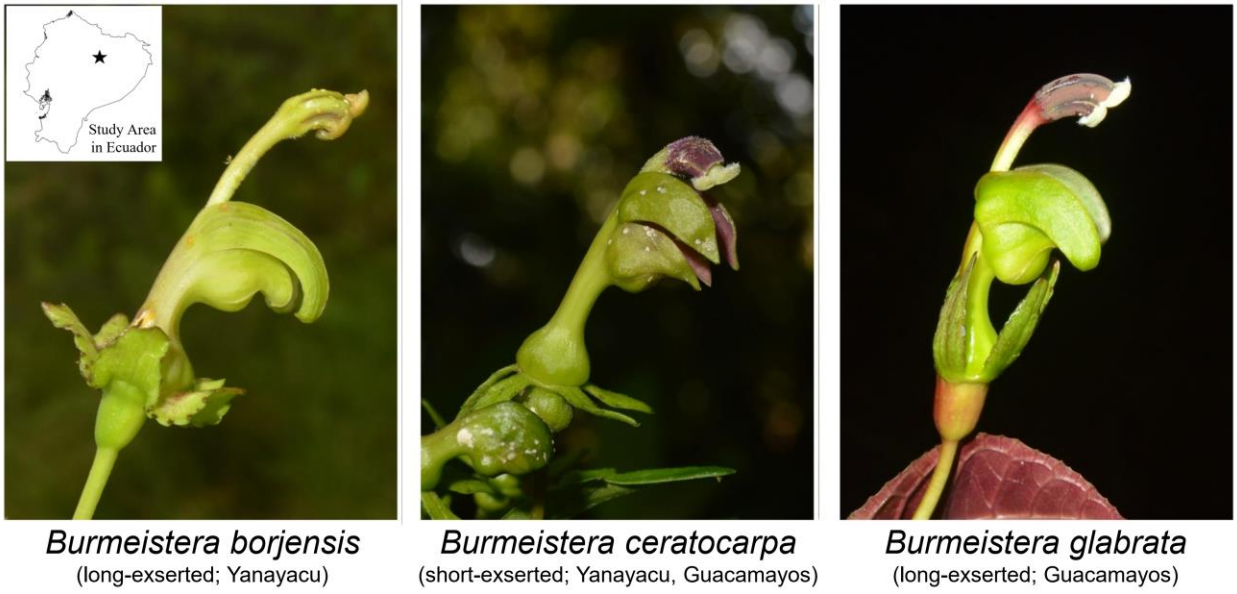

Figure 1. Flowers of three bat-pollinated *Burmeistera* species from two locations used in this study. Flowers of *B. ceratocarpa* have reproductive structures located on a staminal column shortly exserted outside of the corolla tube, resulting in localized pollen deposition on the tip of the snout of its bat pollinators. In contrast, both *B. glabrata* and *B. borjensis* have longer staminal columns able to contact a larger surface area of the bats' heads.

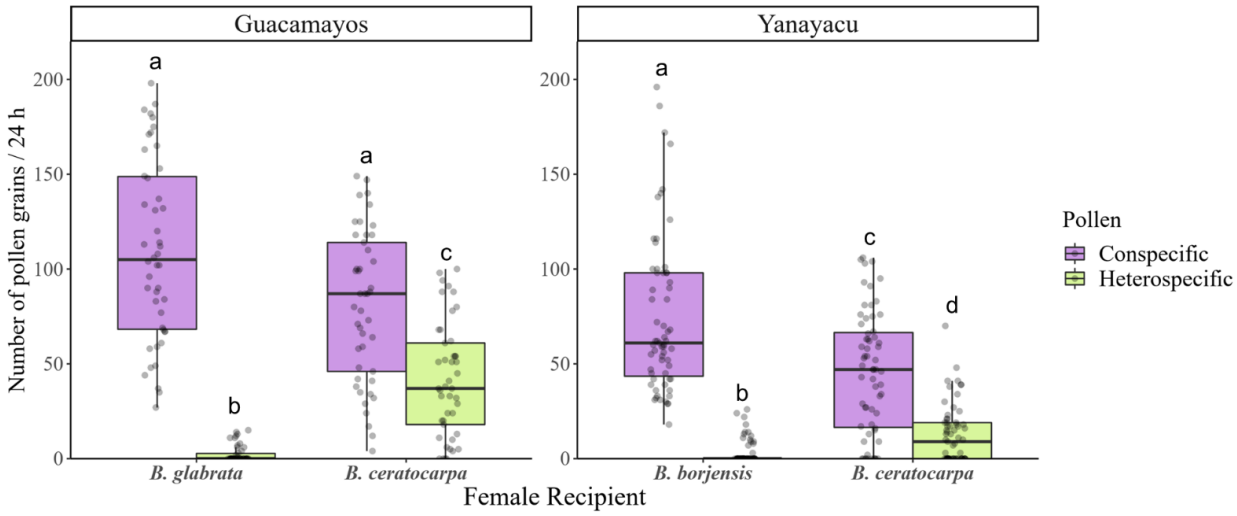

Figure 2. Conspecific and heterospecific pollen deposition over a 24 h period for two species pairs of bat-pollinated *Burmeistera* (Campanulaceae: Lobelioideae) in two cloud forests locations in Ecuador. Lowercase letters above boxplots indicate significant differences between species and pollen deposition type at each location after Bonferroni correction for multiple comparisons.

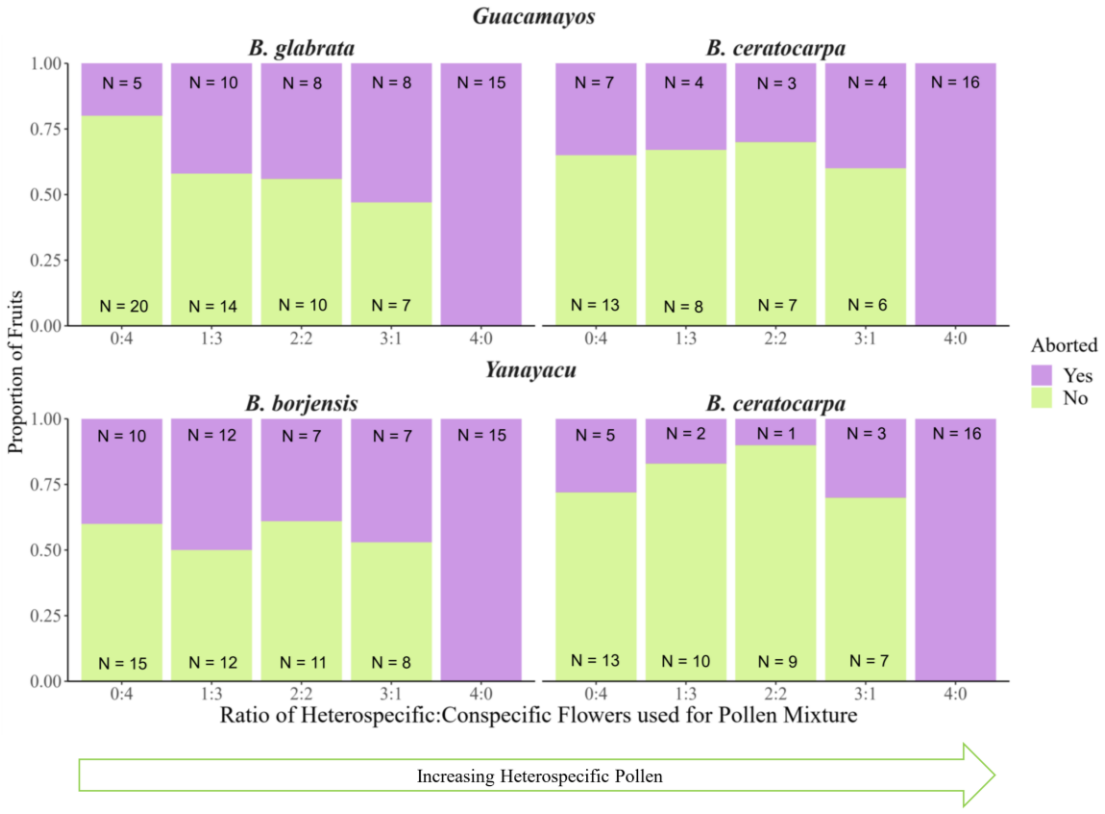

Figure 3. Proportion of matured and aborted fruits across the cross-pollination treatments used to evaluate the effect of increased heterospecific pollen deposition in fruit abortion rates of two *Burmeistera* species pairs from two sites in Ecuador. Pollination treatments were defined by the ratio of heterospecific to conspecific flowers used to make the pollen mixtures that were applied to flowers.

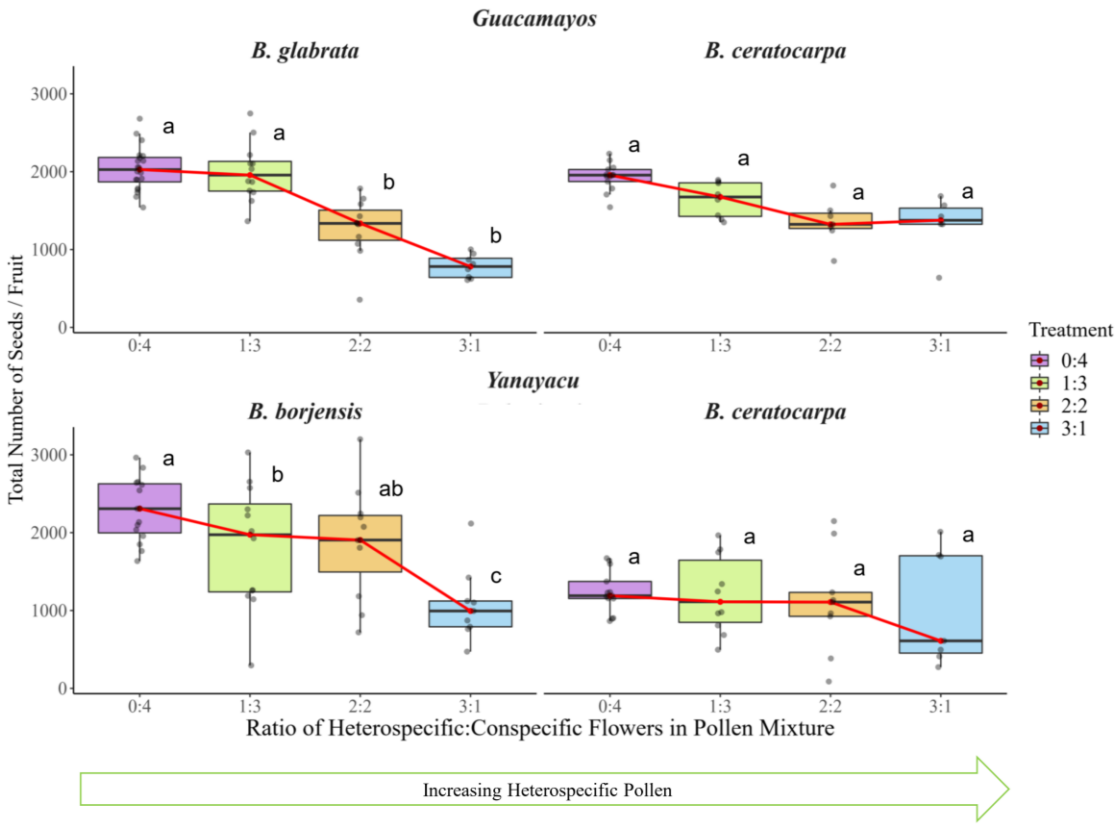

Figure 4. Total number of seeds produced per fruit according to different cross-pollination treatments used to evaluate the effect of increasing heterospecific pollen deposition on seed production in two of *Burmeistera* species pairs from two localities in Ecuador. Pollen mixtures were prepared using different ratios of heterospecific to conspecific flowers, and are arranged from left to right indicating increased heterospecific pollen deposition. Different lowercase letters show significant differences between treatments within each species after correcting for multiple comparisons ($\alpha = 0.05$). A red line linking the median values across treatments is added for visualization purposes only.

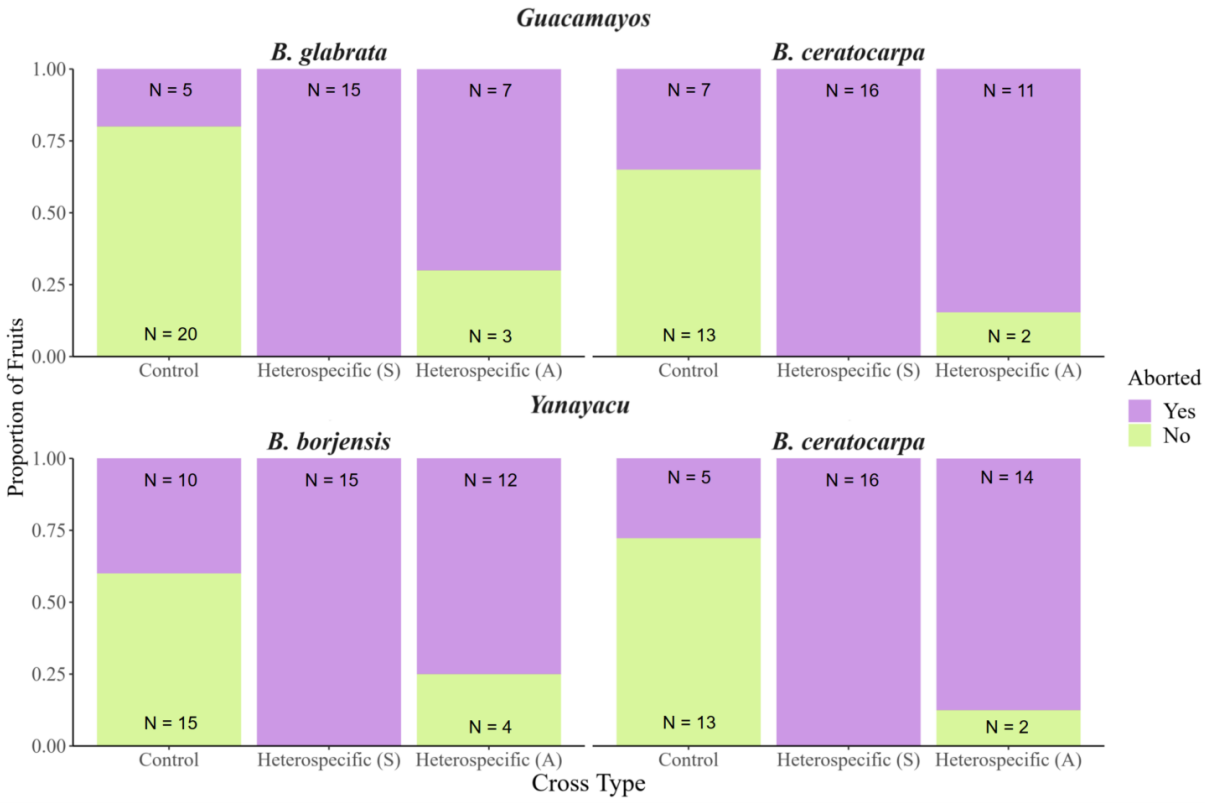

Figure 5. Proportion of matured and aborted fruits from sympatric (S) and allopatric (A) heterospecific crosses in two *Burmeistera* species pairs from two sites in Ecuador.

TABLES

Table 1. Number of hand pollinations performed and fate of the resulting fruits under different conspecific and heterospecific pollen mixture treatments used in two species pairs of bat-pollinated *Burmeistera* from Ecuador.

Site	Species	Ratio of HS:CS flowers used in pollen mixtures	Total no. pollinations performed	Proportion of fruits (*N*)		
				Matured	Aborted	Lost
Guacamayos	*B. glabrata*	0:4	25	0.80 (20)	0.20 (5)	0 (0)
		1:3	24	0.58 (14)	0.42 (10)	0 (0)
		2:2	20	0.50 (10)	0.40 (8)	0.10 (2)
		3:1	15	0.47 (7)	0.53 (8)	0 (0)
		4:0	15	0 (0)	1.00 (15)	0 (0)
	B. ceratocarpa	0:4	20	0.65 (13)	0.35 (7)	0 (0)
		1:3	12	0.67 (8)	0.33 (4)	0 (0)
		2:2	11	0.64 (7)	0.27 (3)	0.09 (1)
		3:1	10	0.60 (6)	0.40 (4)	0 (0)
		4:0	16	0 (0)	1.00 (16)	0 (0)
Yanayacu	*B. borjensis*	0:4	26	0.58 (15)	0.38 (10)	0.04 (1)
		1:3	24	0.50 (12)	0.50 (12)	0 (0)
		2:2	18	0.61 (11)	0.39 (7)	0 (0)
		3:1	15	0.53 (8)	0.47 (7)	0 (0)
		4:0	15	0 (0)	1.00 (15)	0 (0)
	B. ceratocarpa	0:4	18	0.72 (13)	0.28 (5)	0 (0)
		1:3	12	0.83 (10)	0.17 (2)	0 (0)
		2:2	10	0.90 (9)	0.10 (1)	0 (0)
		3:1	11	0.64 (7)	0.27 (3)	0.09 (1)
		4:0	16	0 (0)	1.00 (16)	0 (0)

Table 2. Mixed effects model for the total number of seeds per fruit under different pollination treatments in two *Burmeistera* species pairs from two sites in Ecuador. Linear contrasts within each species are shown by the ratios of heterospecific:conspecific flowers used to make the pollen mixtures that were applied to flowers in the treatment levels being compared.

Negative Binomial Mixed Effects Model for Total Number of Seeds per Fruit					
Random Effects	variance	st.dev			
Plant	6.22E-10	2.49E-05			
Fixed Effects	X^2	df	p. value		
Pollination Treatment	68.842	3	**<0.0001**		
Species	44.459	3	**<0.0001**		
Treatment x Species	17.636	9	**0.0396**		
Contrasts	estimate	SE	df	t.ratio	p.value
Guacamayos					
B. glabrata					
0:4 - 1:3	1.028	0.103	153	0.277	0.9925
0:4 - 2:2	1.649	0.198	153	4.165	**0.0003**
0:4 - 3:1	2.473	0.392	153	5.707	**<0.0001**
1:3 - 2:2	1.604	0.210	153	3.620	**0.0022**
1:3 - 3:1	2.406	0.401	153	5.266	**<0.0001**
2:2 - 3:1	1.499	0.269	153	2.256	0.1131
B. ceratocarpa					
0:4 - 1:3	1.173	0.153	153	1.218	0.6166
0:4 - 2:2	1.428	0.208	153	2.438	0.0742
0:4 - 3:1	1.483	0.232	153	2.515	0.0615
1:3 - 2:2	1.218	0.199	153	1.207	0.6234
1:3 - 3:1	1.265	0.218	153	1.360	0.5262
2:2 - 3:1	1.039	0.192	153	0.206	0.9969
Yanayacu					
B. borjensis					
0:4 - 1:3	1.396	0.153	153	3.040	**0.0146**
0:4 - 2:2	1.286	0.141	153	2.284	0.1062
0:4 - 3:1	2.183	0.308	153	5.535	**<0.0001**
1:3 - 2:2	0.921	0.114	153	-0.666	0.9098
1:3 - 3:1	1.563	0.237	153	2.941	**0.0196**
2:2 - 3:1	1.697	0.258	153	3.479	**0.0036**
B. ceratocarpa					
0:4 - 1:3	1.091	0.161	153	0.592	0.9343
0:4 - 2:2	1.404	0.23	153	2.071	0.1672
0:4 - 3:1	1.477	0.268	153	2.149	0.1424
1:3 - 2:2	1.287	0.225	153	1.443	0.4747
1:3 - 3:1	1.354	0.259	153	1.583	0.3915
2:2 - 3:1	1.052	0.215	153	0.249	0.9946

Table 3. Results from sympatric and allopatric heterospecific crosses between two species pairs of bat-pollinated *Burmeistera* from Ecuador.

Site	Species	Cross Type	No. pollinations performed	Proportion of fruits (*N*)		# Seeds Per Fruit (mean ± SD)
				Matured	Aborted	
Guacamayos	*B. glabrata*	Sympatric Conspecific (control)	25	0.80 (20)	0.20 (5)	2038 ± 282
		Sympatric Heterospecific (*B. ceratocarpa*-G)	15	0 (0)	1.00 (15)	
		Allopatric Heterospecific (*B. ceratocarpa*-Y)	10	0.30 (3)	0.70 (7)	788 ± 202
	B. ceratocarpa	Sympatric Conspecific (control)	20	0.65 (13)	0.35 (7)	1992 ± 245
		Sympatric Heterospecific (*B. glabrata*)	16	0 (0)	1.00 (16)	
		Allopatric Heterospecific (*B. borjensis*)	13	0.15 (2)	0.85 (11)	557 ± 187
Yanayacu	*B. borjensis*	Sympatric Conspecific (control)	25	0.58 (15)	0.38 (10)	2289 ± 406
		Sympatric Heterospecific (*B. ceratocarpa*-Y)	15	0 (0)	1.00 (15)	
		Allopatric Heterospecific (*B. ceratocarpa*-G)	16	0.25 (4)	0.75 (12)	626 ± 115
	B. ceratocarpa	Sympatric Conspecific (control)	18	0.72 (13)	0.28 (5)	1238 ± 272
		Sympatric Heterospecific (*B. borjensis*)	16	0 (0)	1.00 (16)	
		Allopatric Heterospecific (*B. glabrata*)	16	0.13 (2)	0.87 (14)	310 ± 65

CHAPTER 3.

Lack of introgressive gene flow despite extensive interspecific pollen transfer among sympatric bat-pollinated *Burmeistera* (Campanulaceae: Lobeliodeae)

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Abstract

Mounting evidence over the last two decades has established introgression between close relatives as a major evolutionary force across the Tree of Life. In flowering plants, ecological factors such as geographic contact and interspecific pollen transfer by shared pollinators can promote introgression, yet these relationships remain understudied. Young plant clades where sympatry and pollinator sharing are common could be particularly prone to introgression and thus comprise ideal systems to explore the relationship between pollen movement and gene flow between close relatives. We evaluated patterns of interspecific pollen transfer and introgression between six Ecuadorian species of bat-pollinated *Burmeistera* (Campanulaceae), a young Neotropical radiation. Species distribution patterns across three study sites and differences in anther/stigma exsertion allowed us to explore their effects on pollen movement and introgression. Samples of pollen deposited onto stigmas revealed extensive pollen transfer between sympatric

populations of the study species despite differences in exsertion length, with substantial asymmetric pollen transfer from long- to short-exserted species across the three sites. Using D-statistics on a phylogenomic dataset, we tested for introgressive gene flow among sympatric and allopatric populations of the study species but found no significant evidence of introgression in sympatry. A second set of D-tests also showed that introgression has not occurred among *Burmeistera* species with similar floral exsertions despite their high likelihood of interacting via interspecific pollen transfer. Although sympatry and interspecific pollen transfer are prevalent among our *Burmeistera* study species, the lack of detectable introgression suggest that reproductive isolation at the gametic or postzygotic stages is sufficient to prevent gene flow. Our results show that young plant lineages can show limited introgression despite the occurrence of ecological factors that could promote it.

Keywords: pollinator sharing, phylogenetic discordance, reproductive isolation, plant speciation, cloud forest, Andes

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With the rapid advances in molecular phylogenetics over the last two decades, there has been an ever-increasing body of evidence highlighting introgressive hybridization as a major evolutionary force across the Tree of Life (Ellstrand 2014; Mallet et al. 2016; Payseur and Rieseberg 2016; Goulet et al. 2017). Genetic exchange between diverging species can facilitate the transfer of adaptive genetic variation, impose selection towards increased reproductive isolation, or even generate new hybrid lineages. Such transfer of genes across species boundaries can generate conflicting evolutionary relationships that can be inferred from gene and species trees causing phylogenetic discordance (Mallet et al. 2016). Stochastic processes such as incomplete lineage sorting can also generate such phylogenetic conflict (Maddison and Knowles 2006), thus, consideration of potential ecological factors that might promote introgression can shed important insights on its relative importance during diversification.

In flowering plants, both geographic and pollination isolation must be overcome for hybridization to occur as they influence the likelihood of interspecific mating events (Baack et al. 2015; Moreira-Hernández and Muchhala 2019). Therefore, considering ecological factors such as geographic proximity and the occurrence of interspecific pollen transfer between sympatric populations can benefit studies of historical introgression during plant diversification. The geographic context of introgression has been widely assessed by many studies testing different past introgression scenarios between a single species pair (Rieseberg et al. 1999; Minder et al. 2007; Stankowski et al. 2015; Roda et al. 2017). However, fewer studies have done this with multiple species from the same clade (Eaton and Ree 2013; Eaton et al. 2015; Pease et al. 2016; Hamlin et al. 2020), and most have not examined associations between floral differences mediating pollen transfer and patterns of historical introgression (Moreira-Hernández and Muchhala 2019). Two clade-level studies have nonetheless examined the relationship between historical introgression and two other pre-pollination isolation barriers, flowering time and mating system differences, as these two factors also affect the opportunities for interspecific mating. Spriggs et al. (2019) quantified flowering time and introgression between members of the North

American *Lentago* clade of the genus *Viburnum* (Adoxaceae) and found complex patterns of introgression involving multiple species pairs despite significant asynchronous flowering occurring in areas of sympatry. Their result indicates that the observed genetic exchange likely took place before the species evolved their current differences in flowering time. The second study by Hamlin et al. (2020) evaluated the effect of mating system differences on introgression in wild tomatoes (*Solanum*) and found that introgression was more common between species that shared the same mating system and, when it did happen between species with contrasting mating systems, it occurred from more inbreeding to more outbreeding taxa. Their results suggest that mating system can prevent interspecific mating events and limit introgression (Hamlin et al. 2020). Despite these two valuable contributions, it remains unknown whether pollen transfer between sympatric members of a clade sharing the same pollinators can offer any insights on patterns of past introgression.

Recent plant radiations are ubiquitous in tropical and subtropical regions of the world especially at high elevations (Hughes and Atchison 2015; Lagomarsino et al. 2016; Nevado et al. 2018; Vasconcelos et al. 2020), and commonly exhibit high levels of sympatry with multiple species co-occurring together. Although specialization towards different groups of pollinators is frequently observed in many such clades (Abrahamczyk et al. 2014; Lagomarsino et al. 2017; Serrano-Serrano et al. 2017; Dellinger et al. 2019), pollinator sharing and interspecific pollen transfer between relatives can often occur in areas of sympatry generating opportunities for hybridization (Kay 2006; Muchhala 2006; Tong and Huang 2016; Mesquita-Neto et al. 2018). In fact, high rates of hybridization are expected in rapidly diversifying clades as there might not have been sufficient time to evolve complete reproductive isolation as new lineages arose (Givnish 2015). Many phylogenomic studies in recent radiations have indeed shown substantial historical introgression and corresponding phylogenetic conflict (Pease et al. 2016; Loiseau et al. 2021; Scharmann et al. 2021), often in combination with incomplete lineage sorting (Rose et al. 2021; Kandziora et al. 2022). Thus, recent plant radiations where sympatry and pollinator sharing

are frequent offer a great opportunity to explore the interplay between patterns of interspecific pollen transfer and historical introgression during diversification.

The Neotropical bellflowers of the genus *Burmeistera* (Campanulaceae) comprise a monophyletic clade of predominantly bat-pollinated plants within the recent explosive radiation of Andean Lobelioideae (~600 spp; < 5.0 ma; Figure 1; Lagomarsino *et al.*, 2016; Bagley *et al.*, 2020). As in other recent radiations, phylogenomic evidence has shown high levels of phylogenetic discordance between gene and species trees in *Burmeistera* (Bagley et al. 2020), and while incomplete lineage sorting likely contributes to this pattern, several other features of this clade suggest introgression may also have played an important role. First, sympatry is very widespread in *Burmeistera*; its center of diversity is located in the cloud forests of the northwest Andes of Colombia and Ecuador where 4-6 (sometimes up to 8) species can be found together in sympatry at any one site with near complete flowering overlap year-round (Lammers 2002; Muchhala 2006; Knox et al. 2008; Moreno and Muchhala 2011; Garzón-Venegas and González 2012). Second, field, morphological and comparative studies of pollination in *Burmeistera* have confirmed or strongly inferred that approximately 90% of the species are pollinated by small nectar-feeding bats, which are known to frequently carry multi-species pollen loads (Glossophaginae; Muchhala, 2003, 2006; Lagomarsino *et al.*, 2017). Third, analysis of pollen arriving at stigmas confirms high rates of interspecific pollen transfer among co-occurring *Burmeistera* (Muchhala 2006). Fourth, sympatric *Burmeistera* often exhibit differences in anther/stigma exsertion which results in differential pollen placement on the head of the bat pollinators which reduces, but does not completely prevent, pollen transfer between species (Figure 1B; Muchhala & Potts, 2007; Muchhala, 2008). Notably, when pollen transfer occurs between species with different floral exsertions it occurs predominantly from long- to short-exserted species and much less frequently in the opposite direction (Muchhala and Potts 2007). Exsertion length differences are overdispersed against random expectations within *Burmeistera* communities, however, suggesting that they evolved in situ after secondary contact (Muchhala

and Potts 2007). If this is the case, bouts of introgression could have occurred upon secondary contact before sympatric species could evolve their current exsertion length differences to prevent maladaptive introgression. Alternatively, if species were already fully reproductive isolated and did not introgress when they came in contact, exsertion length differences could have still evolved in the absence of gene flow due to selection to prevent reproductive interference (Moreira-Hernández and Muchhala 2019). Distinguishing between these two scenarios would require quantifying patterns of pollen transfer and introgression between sympatric *Burmeistera* species differing in exsertion length. For all of these reasons, *Burmeistera* can be considered an ideal system to study if and how interspecific pollen transfer interactions might reveal patterns of historical introgression and aid our understanding of pollinator-mediated process influencing floral evolution and diversification in the Neotropics.

In this study, we evaluated patterns of interspecific pollen transfer and tested for introgressive gene flow among sympatric and allopatric populations of six bat-pollinated *Burmeistera* species (3 short- and 3 long-exserted) in three cloud forest sites on the eastern Andean slopes of Ecuador. First, we quantified conspecific and heterospecific pollen receipt in stigmas of all *Burmeistera* species present at each site. We wanted to estimate the extent by which species within a site interact via interspecific pollen transfer, predicting that there should be substantial pollen transfer between species with similar exsertions and from long- to short-exserted species as previous work with *Burmeistera* has shown. Second, we used targeted sequence capture of 561 low copy nuclear loci to build a population-level phylogeny of our study species to serve as a baseline for our introgression analyses. Third, we used D-statistics (Durand et al. 2011; Eaton and Ree 2013) to infer introgression between sympatric and allopatric populations of our study species in two ways. We carried out different sets of D-statistic tests to evaluate whether more introgression is observed: 1) between sympatric populations of the study species, and 2) between species with similar floral exsertions that are more likely to interact via interspecific pollen transfer.

MATERIALS AND METHODS

Study system and populations. — *Burmeistera* H. Karst. & Triana (Campanulaceae: Lobelioideae) is a Neotropical genus of ~120 species of herbs, terrestrial and hemiepiphytic shrubs distributed from Guatemala to northern Peru (Figure 1A; Lammers, 2007; Knox *et al.*, 2008; Lagomarsino *et al.*, 2014). Flowers are bilaterally-symmetrical and protandrous, with bell-shaped tubular corollas and a single staminal column at the tip of which the stigma and the anthers are located (Figure 1A). The distance separating the tip of the staminal column and the corolla constriction determines the exsertion of the reproductive parts relative to the head of the bat pollinators during flower visitation, ultimately defining the precise location where pollen will be deposited on the head of the animals (Figure 1B; Muchhala, 2006, 2008; Muchhala & Potts, 2007). Most *Burmeistera* species can be categorized as either short- (<20 mm) or long-exserted (>20 mm), and these differences minimize pollen transfer between species (Muchhala and Potts 2007; Muchhala 2008), which still occurs between species with similar exsertions and also asymmetrically from long- to short-exserted species (Muchhala 2006; Muchhala and Potts 2007).

To evaluate patterns of interspecific pollen transfer and introgression between sympatric *Burmeistera* we conducted fieldwork with populations from six *Burmeistera* species from three cloud forest sites near the town of Cosanga, Napo Province, Ecuador (Figure 1C). Study sites are located ~7-10 km apart from each other and have similar *Burmeistera* assemblages with small differences in relative densities and in the identity and number of long-exserted species present in each site. Cordillera de los Guacamayos (0°37’22” S, 77°50’26” W; hereafter Guacamayos) is a public access trail along a forested ridge at 2250 masl within the large Antisana Ecological Reserve (1200 km^2). It sustains very humid mountain cloud forest along the Amazon-facing slopes east of the Cosanga River Valley. The second location, Sierra Azul (0°40’25” S, 77°55’32” W) is a private biological reserve bordering Antisana to the west but located within the Cosanga River Valley at the end of an old unpaved road southwest of the near town of Cosanga.

It has a mixture of mature cloud forest with second growth and cattle pastures. Yanayacu Biological Station (0°36'03" S, 77°53'22" W; hereafter Yanayacu) is a small private reserve also bordering Antisana inside the Cosanga River Valley but about 7 km north of Sierra Azul and much closer to the town and surrounded by farmland and areas of second growth. It has a mixture of abandoned pastures with second growth and small pockets of mature forest along ridgetops. The short-exserted *B. ceratocarpa*, *B. sodiroana*, and *B. succulenta* are found in all three sites, whereas the long-exserted *B. borjensis* occurs in Sierra Azul and Yanayacu, *B. sierrazulensis* occurs in Sierra Azul only, and *B. glabrata* is exclusive to Guacamayos (Figure 1C). These differences in species composition between sites allowed us to study patterns of interspecific pollen transfer and introgression between sympatric and allopatric populations of short- and long-exserted bat-pollinated *Burmeistera*.

Quantifying rates of interspecific pollen transfer. — Using methods previously used in *Burmeistera* (Muchhala 2003, 2006), we quantified nightly receipt of conspecific and heterospecific pollen on stigmas of the 6 study species in our study sites. We wrapped up staminal columns of flowers in the field with a thin parafilm layer and we placed a small rectangle (0.5 x 0.8 cm) of clear double-sided tape in the position of the stigma at the tip of the column. We collected the tape samples 24 hours afterwards, placed them in microscope slides, and covered them with clear single-sided tape. Although the tapes were left for 24 h on the flowers we expect the pollen samples to reflect primarily nightly pollen deposition by bats during the first 8-12 h as pollen deposition by hummingbirds and other diurnal floral visitors is negligible (Muchhala 2006). We stained the pollen samples with fuchsin gelatin cubes and observed them under a microscope to identify and count all pollen grains found along two perpendicular transects passing through the center of the tape rectangle. The stained pollen grains could be identified to species due to differences in grain size and the shape of the colpii.

From these pollen counts we estimated conspecific pollen receipt for each of our study species in each site, as well as heterospecific pollen receipt from the other co-occurring

Burmeistera species. We evaluated differences in conspecific and heterospecific pollen receipt between species found at each site using a generalized linear mixed model with a negative binomial distribution specifying species and type of pollen receipt (conspecific or heterospecific) as fixed factors and the identity of the plant bearing the flower as the random factor. For each of the species we pooled together all heterospecific pollen grains received regardless of their identity because the very low counts for some of them prevented their individual consideration in these analyses. This metric gives us an idea of the relative exposure to pollen from sympatric relatives and thus potential opportunities for introgression.

Genomic DNA extraction, high throughput sequencing, and bioinformatics. — We collected silica-dried leaf tissue from our species in the field to conduct DNA genomic extractions from a total of 116 individual samples plus an outgroup (*B. xerampelina*; Table 1). We performed the targeted hybrid enrichment sequencing approach developed for *Burmeistera* described in Bagley et al. (2020) to build genomic datasets for downstream phylogenetic and introgression analyses. After extracting total genomic DNA using the CTAB method (Doyle and Doyle 1987), DNA libraries were prepared and sequenced by RAPiD Genomics (Gainesville, FL) on an Illumina HiSeq 3000 sequencer plataform. We filtered out short and low quality reads (PHRED scores < Q20) after removing the Illumina adapters before loci assembly following the HybPiper v1.3.1 (Johnson et al. 2016) pipeline as described in Bagley et al. (2020). In total we retrieved 561 loci with 50% occupancy for phylogeny estimation and 502 loci with 100% sampling completeness for our introgression analyses.

Phylogenetic analyses. — We built a maximum likelihood population-level phylogeny for our six study species from the 561 nuclear loci dataset using IQ-TREE v2.0.3 (Nguyen et al. 2015). We used the ¨completeConcatSeqs¨ function of PIrANHA (Bagley 2020) to generate a supermatrix of all 561 loci alignments and partition block files to feed into IQ-TREE for phylogeny estimation. Best-fit parameters and appropriate evolutionary models for tree

estimation were calculated using ModelFinder and percent nodal support was obtained by 1000 ultrafast bootstraps pseudoreplicates based on the GTR+ nucleotide substitution model (Kalyaanamoorthy et al. 2017; Hoang et al. 2018). We included the species *B. xerampelina* as outgroup, as it is inferred to be sister to all other *Burmeistera* species (Bagley et al. 2020).

Introgression analyses. — The four taxon D-statistic was developed to infer introgression between divergent lineages based on counts of biallelic single nucleotide polymorphisms that contradict the known species tree topology (Durand et al. 2011; Eaton and Ree 2013). Given a four taxon tree of the form (P1,P2),P3),O) where the ancestral allele is defined as A and the derived allele is defined as B, the D-statistic test compares the counts of the discordant patterns ABBA and BABA. These two patterns represent instances where the derived alleles are shared between P2-P3 and P1-P3, respectively, contradicting the tree topology. These patterns are expected to be generated in equal frequencies due to the random sorting of ancestral polymorphisms (i.e. incomplete lineage sorting). However, if introgression has occurred between P3 and either P1 or P2, either BABA or ABBA patterns might occur with higher frequency, respectively. The D-statistic test provides a way to distinguish between these two scenarios by estimating the asymmetry in the relative numbers of these two discordant patterns against the null hypothesis of no introgression (i.e. D equals zero). An excess of ABBA patterns will result in positive D values indicating introgression between P2-P3, whereas an excess of BABA patterns will result in negative D values supporting genetic exchange between P1-P3.

We used these D-statistics to test for specific introgression scenarios involving our *Burmeistera* study species. First, we used a set of D-statistic tests to infer introgression between the different populations of short-exserted species from the three study sites and their long-exserted relatives. Each of these tests included two sympatric samples of different short-exserted species from one of the sites and a third sample from one of the long-exserted species (sympatric or not). In total, we performed 9 of these tests, reflecting the three possible pairings of sympatric short-exserted species from each our three study sites. We performed two groups of replicates for

each test, one including all samples from long-exserted species (sympatric and allopatric) and the other only including sympatric samples. This allowed us to compare whether a possible signal of introgression between long and short-exserted species would be greater when only sympatry samples were used in the test.

We designed a second set of D-statistic tests to evaluate introgression within the short- and long-exserted groups of *Burmeistera* study species. The goal of these tests was to determine if species with similar floral exsertions likely to engage in interspecific pollen transfer interactions would carry a detectable signal of introgression. To do this, we structured different test types considering all possible geographic combinations. For the short-exserted species which are present in all three sites, we performed five groups of tests: all sympatric samples for the three ingroup taxa, only P1-P2 sympatric, only P1-P3 sympatric, only P2-P3 sympatric, and all three ingroup taxa allopatric. For long-exserted species, we only had *B. borjensis* and *B. sierrazulensis* co-occurring together in Sierra Azul so we performed tests where the samples used from these two species were sympatric (i.e. from Sierra Azul) and another group of tests where samples from all three long-exserted species were allopatric thus only including the *B. borjensis* samples from Yanayacu.

Each of the D-statistic tests described above was performed the following way. From the 502 loci alignments with complete sampling coverage (including the outgroup), we subsampled single random biallelic SNPS from each loci using the program snp-sites (https://github.com/sanger-pathogens/snp-sites) and concatenated these into a supermatrix using the ¨completeConcatSeqs¨ function of PIrANHA (Bagley 2020). The resulting matrix was used to perform D-statistic tests using the software Comp-D (Mussmann et al. 2020). Each test was performed iterating exhaustively over each sampled individual from the chosen ingroup taxa to generate individual test replicates. *D* values for each replicate were calculated via bootstrapping the 502 SNPs loci with replacement (1000 iterations) and then transformed into Z-scores to obtain two-tailed *p*-values using $\alpha = 0.01$ as the significance threshold after correcting for multiple

comparisons. A global Z-score was also calculated from the distribution of D values across all replicates for each test and its significance was assessed against the null hypothesis of no introgression (i.e. mean D equals zero; Eaton & Ree, 2013; Eaton *et al.*, 2015; Hamlin *et al.*, 2020).

RESULTS

Interspecific pollen transfer in sympatry. — Samples of pollen receipt revealed that in all three sites the majority of pollen received per flower per night by the study species was conspecific but interspecific pollen transfer between them was still common (Figure 2). Overall conspecific pollen receipt was significantly higher than that of heterospecific pollen across the study species in each of the three sites (Likelihood ratio tests: Guacamayos: $X^2 = 152.25$, $P < 0.0001$; Yanayacu: $X^2 = 28.69$, $P < 0.0001$; Sierra Azul: $X^2 = 161.19$, $P < 0.0001$; Figure 2). We also detected significant species differences in conspecific and heterospecific pollen receipt in Guacamayos and Sierra Azul (Likelihood ratio tests: Guacamayos: $X^2 = 47.99$, $P < 0.0001$; Sierra Azul: $X^2 = 30.83$, $P < 0.0001$), but not in Yanayacu (Likelihood ratio test: $X^2 = 0.70$, $P = 0.8742$). However, significant interactions between species and type of pollen deposition (conspecific or heterospecific) were found in all three sites (Likelihood ratio tests: Guacamayos: $X^2 = 116.12$, $P < 0.0001$; Yanayacu: $X^2 = 221.62$, $P < 0.0001$; Sierra Azul: $X^2 = 21.63$, $P = 0.0002$; Figure 2). In Guacamayos, *B. glabrata*, *B. ceratocarpa* and *B. huacamayensis* experienced significantly greater receipt of conspecific compared to heterospecific pollen, but there was no significant difference in *B. sodiroana* (Figure 2A). At Yanayacu, *B. borjensis* was the only species that received significantly more conspecific than heterospecific pollen while in the other three species (*B. ceratocarpa*, *B. huacamayensis*, and *B. sodiroana*) the differences were not significant (Figure 2B). Similarly, in Sierra Azul all species showed significantly greater conspecific pollen receipt compared to heterospecific pollen receipt (Figure 2C). Across all three locations between 13-93% of all grains received by the three short-exserted species were from their sympatric long-exserted

relatives. Although receipt of conspecific pollen significantly surpassed that of heterospecific pollen for most species, our data shows that there is still substantial pollen movement amongst the study species in all three study sites providing opportunities for interspecific gene flow.

Phylogenetic relationships. — Our maximum likelihood phylogenetic tree showed well supported relationships between the study species as a baseline for our introgression analyses (Figure 3). Our phylogeny showed *Burmeistera ceratocarpa* as sister to the rest of the study species, which are in turn divided into two subclades: one including *B. huacamayensis* and *B. sodiroana* and the other including the three long-exserted species. Among these three, *B. glabrata* is sister to *B. borjensis* and *B. sierrazulensis* (Figure 3). All but two species were monophyletic, with the exception being that some samples of *B. sierrazulensis* and *B. borjensis* from Sierra Azul interdigitate (Figure 3). Notably, the three species with short-exserted flowers which are present in all three study sites (*B. ceratocarpa*, *B. huacamayensis*, and *B. sodiroana*) exhibited extensive interdigitation at the population level, indicating that gene flow between their populations is high (Figure 3).

Introgression tests between species differing in floral exsertion length. — Overall, we found no significant evidence of introgression between the study species across the three study sites. D-statistic tests between short- and long-exserted species did not reveal introgression signals between both groups of species, not even for tests performed using only samples from sympatric populations (Table 2; Figure 4). D values were not significantly different from zero in all tests performed that used both sympatric and allopatric samples. The sympatric tests tended to exhibit D values deviating slightly from zero, but these were also not significant. Most tests seemed to show different trends depending on the populations used. The tests including *B. huacamayensis* (P1) and *B. ceratocarpa* (P3) had D estimates very close to zero in Guacamayos and Yanayacu, but the replicates using Sierra Azul samples had higher positive values suggestive of low introgression between *B. ceratocarpa* from this site and long-exserted species (P2; Figure 4). Similarly, the tests including *B. sodiroana* (P1) and *B. ceratocarpa* (P3) exhibited lower

negative D values in Guacamayos than in Sierra Azul or Yanayacu (Figure 4), suggestive of some low degree of introgression between these two species at this site. Lastly, the tests including *B. huacamayensis* (P1) and *B. sodiroana* (P2) were the only ones that showed a somewhat consistent pattern across sites exhibiting negative D-values suggesting introgression between *B. huacamayensis* and long-exserted species (P3), although this pattern was weaker for the Guacamayos tests compared to those from Sierra Azul and Yanayacu (Figure 4). Despite this, none of the estimated D values were significantly different from zero and thus we conclude that there is no significant introgression between our *Burmeistera* study species with differing floral exsertions despite extensive interspecific pollen transfer in sympatry.

Introgression tests between species with similar floral exsertion length. — Our D-statistics tests among species with similar floral exsertions did not find statistical support to reject the null hypothesis of no introgression (Table 3; Figure 5). Within the short-exserted species, our results hinted at low levels of introgression between *B. sodiroana* (P2) and *B. ceratocarpa* (P3) that seemed consistent regardless of the geographic origin of the samples used in the tests (Figure 5A). In contrast, amongst the long-exserted species our results suggest a weak signal of introgression between *B. sierrazulensis* (P2) and *B. glabrata* (P3) that seemed slightly stronger when the samples of the third species (*B. borjensis*) used in the test were not sympatric with the former (Figure 5B). The lack of statistical support for these trends, however, indicates that no significant introgression has occurred between our *Burmeistera* study species with similar exsertions either.

DISCUSSION

By estimating patterns of pollen receipt and introgression, we demonstrate that there is no significant signature of past introgressive gene flow among our sympatric *Burmeistera* populations despite extensive levels of interspecific pollen transfer. Although most species received more conspecific than heterospecfic pollen per flower per night, pollen from

heterospecific *Burmeistera* was present in most samples and often accounted for a large proportion of the total pollen receipt. Species with long-exserted flowers received very little heterospecific pollen from other *Burmeistera* while receipt of conspecific and heterospecific *Burmeistera* pollen was very similar in short-exserted species. We also observed high pollen transfer from long- to short-exserted species, indicating that exsertion length differences are not sufficient to prevent all interspecific pollen transfer but also commonly result in asymmetric pollen flow from the former to the latter. Despite patterns of pollen transfer following our expectations, our D-statistic analyses did not detect significant introgression between short-exserted species and their sympatric long-exserted relatives. In addition, our second set of analyses showed that introgression has not occurred between species with similar exsertions either, suggesting that isolating barriers acting during post-pollination stages have been effective at limiting past gene flow. Taken together, our results indicate that opportunities for interspecies gene flow provided by geographic contact and interactions via pollinators do not always result in detectable introgression in rapidly diversifying clades. This study has important implications for our understanding of the consequences of plant-pollinator interactions and on the relative role of introgression and incomplete lineage in rapid plant radiations.

Interspecific pollen transfer and pollinator sharing in rapid radiations. — There has been widespread interest in the study of pollinator shifts and pollination syndrome evolution during the diversification of many plant clades (Kay et al. 2005; Smith et al. 2008; Abrahamczyk et al. 2014; Givnish et al. 2014; Lagomarsino et al. 2017; Dellinger et al. 2019), but taxa that have diversified in the absence of obvious pollinator shifts have received comparatively less attention (Ellis and Anderson 2012). Such clades represent useful systems to study speciation and introgression in the absence of significant pre-pollination isolation, as shared pollinators would be able to provide ample opportunities for genetic exchange during early divergence. We found extensive interspecific pollen transfer between our *Burmeistera* study species despite floral differences that mitigate it, which suggest a high potential for hybridization and introgression. That pollen

transfer occurred mostly among the short-exserted species and asymmetrically from long- to short-exserted species generates testable patterns of expected introgression between sympatric species that do interact via pollen transfer. Yet, we did not find evidence of such introgression scenarios and thus we conclude that there is no relationship between patterns of pollen and gene exchange in the *Burmeistera* communities that we studied.

The lack of introgression in *Burmeistera* that we observed has implcations for the evolution of pollinator-mediated isolation via anther/stigma exsertion. Interspecific pollen transfer by bats can still entail important evolutionary costs for sympatric *Burmeistera* species through reproductive interference. Pollen misplaced onto heterospecific stigmas is one of the major mechanisms that reduce male fitness in angiosperms (Muchhala and Thomson 2012; Minnaar et al. 2019), while heterospecific pollen receipt can interfere with conspecific pollination and affect female fitness (Morales and Traveset 2008; Moreira-Hernández and Muchhala 2019). Previous work in *Burmeistera* attributed the evolution of exsertion length differences between sympatric species to the high male fitness costs from pollen misplacement (Muchhala and Potts 2007; Muchhala and Thomson 2012). Given that we did not detect past introgression between our study species despite widespread interspecific pollen transfer in sympatry, we can reasonably assume that current exsertion length differences probably did not evolve to prevent maladaptive hybridization when our study species came in geographic contact. Instead, our data supports the initial interpretation of exsertion length evolving to avoid reproductive interference between sympatric species due to pollen misplacement and heterospecific pollen deposition by shared bat pollinators (Muchhala and Potts 2007). Overdispersion in floral traits mediating pollen transfer and pollination efficiency has been observed in other plant radiations (Armbruster et al. 1994; Eaton et al. 2012; Newman and Anderson 2020), but the question of whether such pattern arises to prevent maladaptive hybridization or to avoid reproductive interference between already fully isolated species deserves further study.

Barriers to introgression and phylogenetic conflict during rapid diversification. — Even though pollinator sharing and geographic overlap are common in *Burmeistera*, we did not detect any signals of introgression between our study species. Although we sampled a small proportion of the total *Burmeistera* diversity (~130 spp) by including multiple individuals from different locations with slightly different species composition and explicitly comparing sympatric versus allopatric samples in our D-statistic tests, we should have been able to recover a signal of past introgression if one had been present amongst our study populations.

Given that pre-pollination isolation is weak in *Burmeistera*, isolating mechanisms during the gametic (i.e. post-pollination prezygotic) and postzygotic stages must be responsible for preventing gene flow between sympatric species. Cross-pollination experiments with multiple *Burmeistera* species indeed show that crosses between sympatric populations fail to develop fruits and seeds while allopatric crosses often do produce them, although most seeds are aborted early (see Chapter 2 and Chapter 4). Combined with the results we present here, it seems likely that in *Burmeistera* postzygotic isolation evolved first in geographic isolation with gametic isolation evolving later after secondary contact. Postzygotic isolation alone is often sufficient to limit gene flow during early divergence (Coughlan and Matute 2020; Ostevik et al. 2021), and thus the simplest scenario for the patterns we observed in *Burmeistera* would consist on populations coming in contact after intrinsic postzygotic barriers had already evolved that would have made hybridization maladaptive and introgression unlikely. Then later, gametic barriers could have evolved alongside exsertion length differences to reduce the female fitness costs of heterospecific pollen deposition, germination, tube growth, and potential ovule usurpation (Ashman and Arceo-Gómez 2013; Moreira-Hernández and Muchhala 2019).

Our results with a small sample of *Burmeistera* species show that pre-pollination barriers are very weak and not the main mechanism preventing gene flow in this group, which suggest that they must have evolved more recently than postzygotic or gametic barriers. These results also support a limited role of introgression in creating the phylogenetic discordance between gene and

species trees observed in previous phylogenetic studies of *Burmeistera* (Uribe-Convers et al. 2017; Bagley et al. 2020). Therefore, if historic introgression has been rare during the diversification of the genus, then incomplete lineage sorting should probably be the main process driving phylogenetic conflict in this group. The accelerated rhythm of species accumulation in rapid radiations grants little time for ancestral genetic variation to sort neatly amongst diversifying lineages (Maddison and Knowles 2006; Degnan and Rosenberg 2009; Kandziora et al. 2022), and such dynamics could very well have taken place during the evolutionary history of *Burmeistera*. The complex history of Andean uplift and environmental fluctuations during glaciation cycles known to have spurred diversification of *Burmeistera* and its sister lobelioid genera *Centropogon* and *Syphocampylus* (Lagomarsino et al. 2016), as well as many other Andean radiations (e.g. Pérez-Escobar et al. 2017; Nevado et al. 2018), could have produced conditions favoring incomplete lineage sorting by the rapid generation of geographically isolated populations without sufficient time for ancestral variation to follow the splitting of newly created lineages. Elucidating the complex history of lineage diversification in the Andes biodiversity hotspot requires further study of the role of introgression and incomplete lineage sorting, as well as the potential factors that mediate their effects on phylogeny.

Conclusions. — This study demonstrates that quantifying patterns of interspecific pollen transfer combined with systematic tests of introgression across multiple species and populations can shed light on the evolutionary history of recent plant radiations. Failing to detect significant introgression in the face of high levels of pollinator sharing and pollen transfer shows that the role of introgression generating phylogenetic discordance during the diversification of *Burmeistera* was probably limited compared to incomplete lineage sorting. Moreover, post-pollination barriers restricting gene flow between *Burmeistera* species must have arisen remarkably quickly before secondary contact, even in a clade part of one of the fastest plant radiations uncovered yet (550 spp; <5 ma; Lagomarsino et al. 2016). We propose that the role of introgression might be limited in young plant lineages under certain diversification scenarios, even despite the occurrence of

ecological factors that could promote it such as extensive pollinator sharing and interspecific pollen transfer between sympatric populations.

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FIGURES

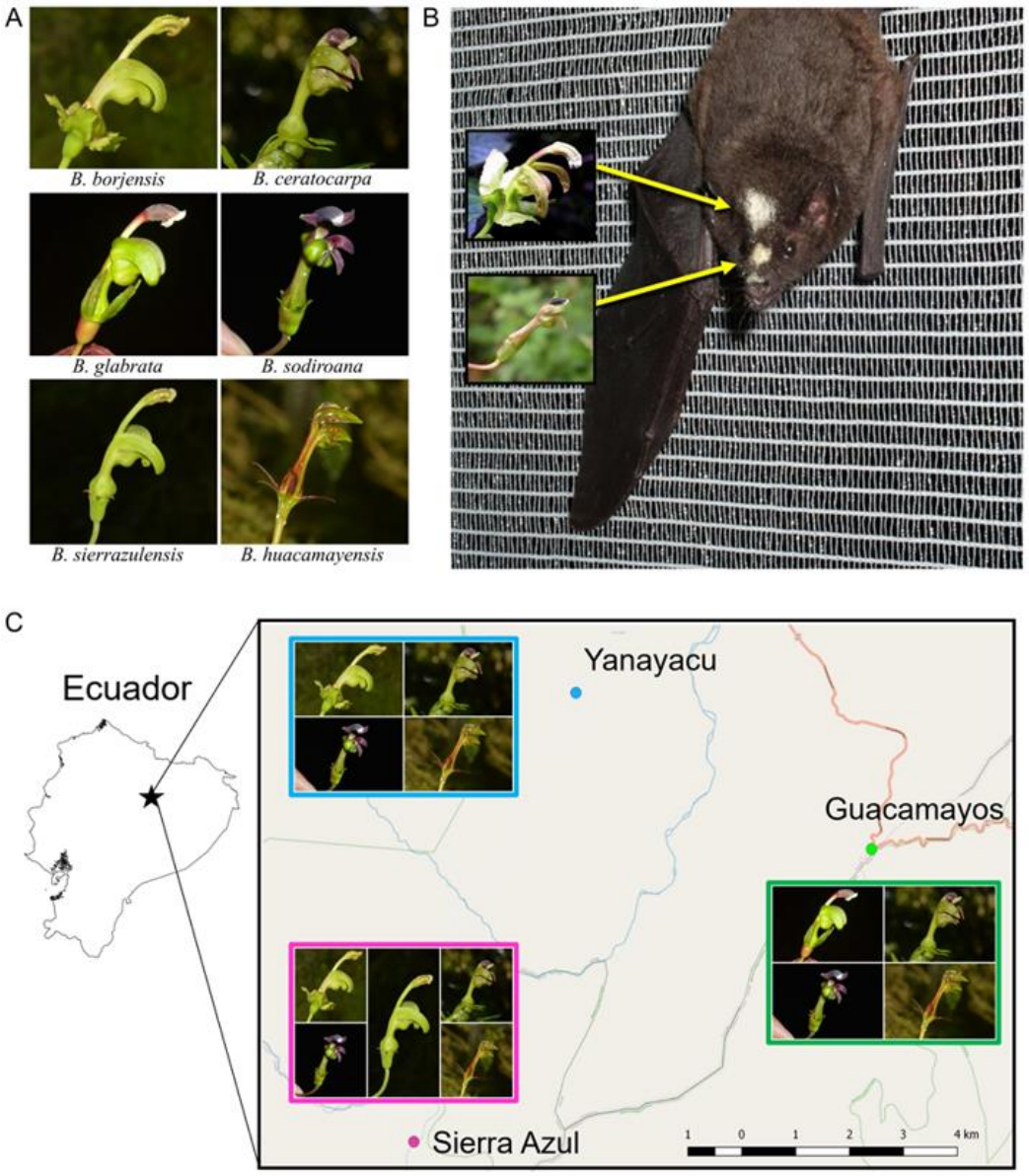

Figure 1. Bat-pollinated *Burmeistera* species and locations used in this study. **A)** Flowers of the six study species showing differences in the length of the staminal tube bearing the exserted reproductive parts (exsertion length). *Burmeistera borjensis*, *B. glabrata*, and *B. sierrazulensis* all have long-exserted flowers, whereas *B. ceratocarpa*, *B. huacamayensis* and *B. sodiroana* have short-exserted flowers. **B)** Short-exserted *Burmeistera* flowers place pollen on the tip of the bats' snout, meanwhile long-exserted flowers deposit pollen further back along the head. Differences in

exsertion length minimize pollen transfer between sympatric *Burmeistera* species but do not prevent it completely. **C)** Distribution of the study species across the three study sites in central Ecuador following the images and names from **A**. Each site only has 1-2 species with long-exserted flowers but the three short-exserted species are found in all three locations.

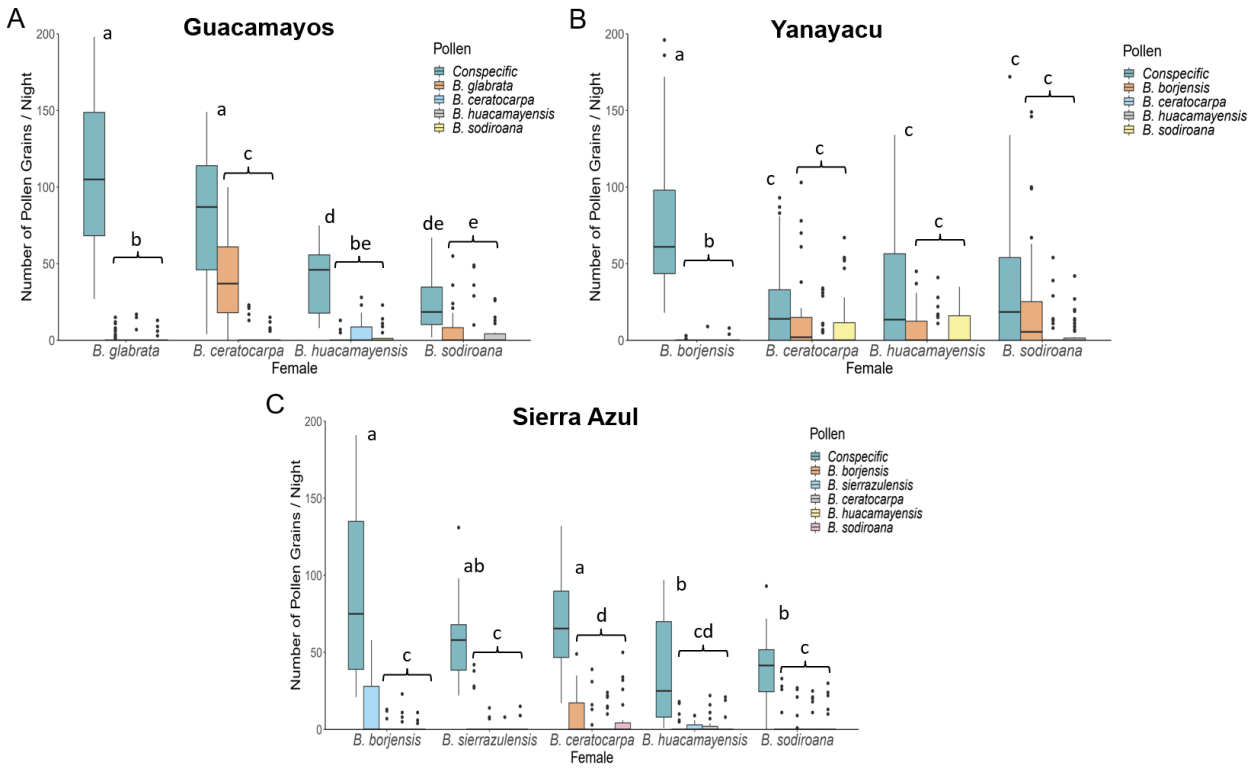

Figure 2. Nightly conspecific and heterospecific pollen receipt in five species of bat-pollinated *Burmeistera* bellflowers (Campanulaceae: *Burmeistera*) from three cloud forest locations in Ecuador. Conspecific pollen receipt is shown first for each species, followed by pollen received from other *Burmeistera* species from the same location. Lowercase letters above individual boxplots and boxplot groups indicate significant differences between conspecific and heterospecific pollen receipt within a species (pooling all heterospecific pollen grains together regardless of their identity) after adjusting *P*-values for multiple comparisons with Bonferroni corrections.

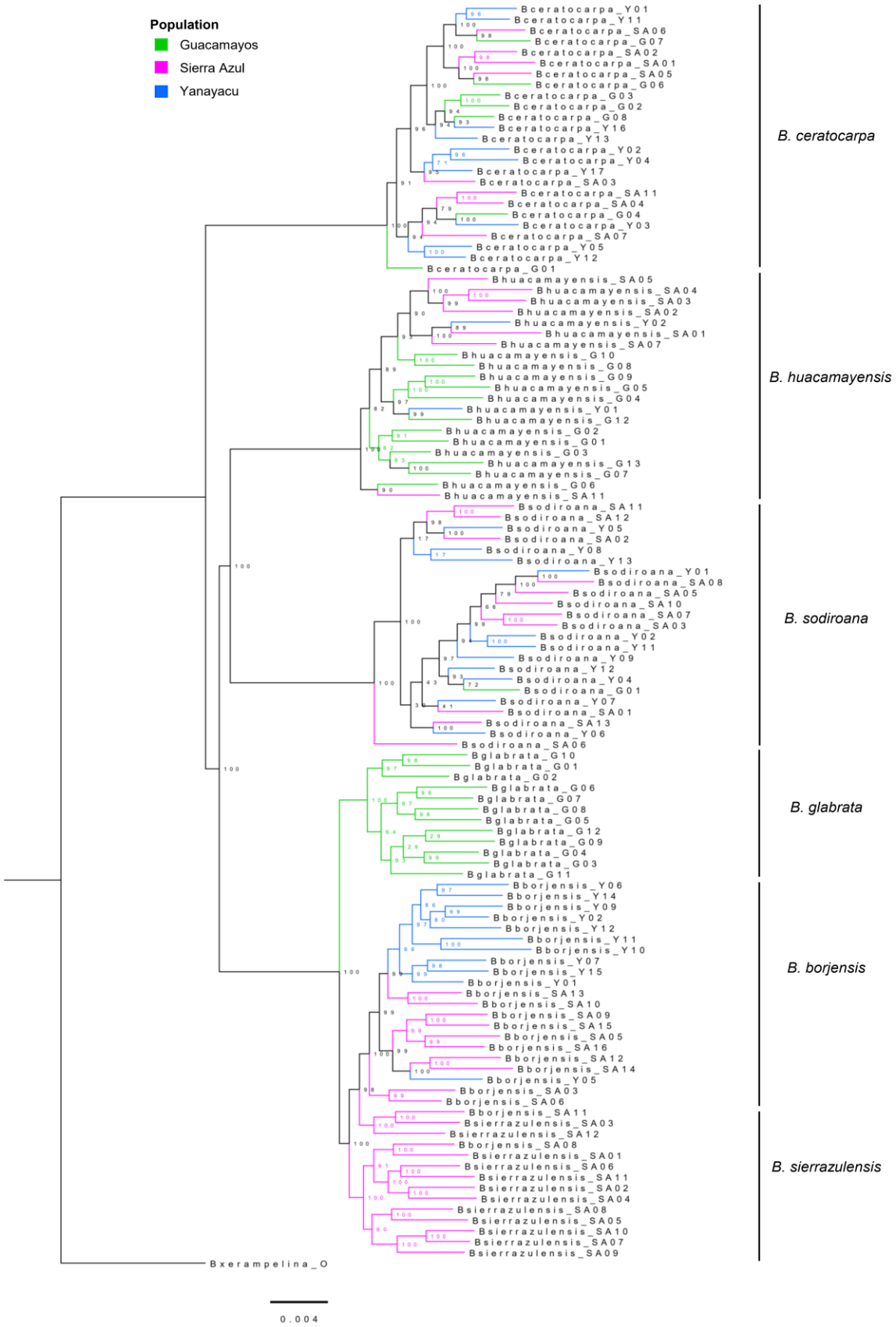
Population
Guacamayos
Sierra Azul
Yanayacu
Bceratocarpa_Y01
Bceratocarpa_Y11
Bceratocarpa_SA06
Bceratocarpa_G07
Bceratocarpa_SA02
Bceratocarpa_SA01
Bceratocarpa_SA05
Bceratocarpa_G06
Bceratocarpa_G03
Bceratocarpa_G02
Bceratocarpa_G08
Bceratocarpa_Y16
Bceratocarpa_Y13
Bceratocarpa_Y02
Bceratocarpa_Y04
Bceratocarpa_Y17
Bceratocarpa_SA03
Bceratocarpa_SA11
Bceratocarpa_SA04
Bceratocarpa_G04
Bceratocarpa_Y03
Bceratocarpa_SA07
Bceratocarpa_Y05
Bceratocarpa_Y12
Bceratocarpa_G01
Bhuacamayensis_SA05
Bhuacamayensis_SA04
Bhuacamayensis_SA03
Bhuacamayensis_SA02
Bhuacamayensis_Y02
Bhuacamayensis_SA01
Bhuacamayensis_SA07
Bhuacamayensis_G10
Bhuacamayensis_G08
Bhuacamayensis_G09
Bhuacamayensis_G05
Bhuacamayensis_G04
Bhuacamayensis_Y01
Bhuacamayensis_G12
Bhuacamayensis_G02
Bhuacamayensis_G01
Bhuacamayensis_G03
Bhuacamayensis_G13
Bhuacamayensis_G07
Bhuacamayensis_G06
Bhuacamayensis_SA11
Bsodiroana_SA11
Bsodiroana_SA12
Bsodiroana_Y05
Bsodiroana_SA02
Bsodiroana_Y08
Bsodiroana_Y13
Bsodiroana_Y01
Bsodiroana_SA08
Bsodiroana_SA05
Bsodiroana_SA10
Bsodiroana_SA07
Bsodiroana_SA03
Bsodiroana_Y02
Bsodiroana_Y11
Bsodiroana_Y09
Bsodiroana_Y12
Bsodiroana_Y04
Bsodiroana_G01
Bsodiroana_Y07
Bsodiroana_SA01
Bsodiroana_SA13
Bsodiroana_Y06
Bsodiroana_SA06
Bglabrata_G10
Bglabrata_G01
Bglabrata_G02
Bglabrata_G06
Bglabrata_G07
Bglabrata_G08
Bglabrata_G05
Bglabrata_G12
Bglabrata_G09
Bglabrata_G04
Bglabrata_G03
Bglabrata_G11
Bborjensis_Y06
Bborjensis_Y14
Bborjensis_Y09
Bborjensis_Y02
Bborjensis_Y12
Bborjensis_Y11
Bborjensis_Y10
Bborjensis_Y07
Bborjensis_Y15
Bborjensis_Y01
Bborjensis_SA13
Bborjensis_SA10
Bborjensis_SA09
Bborjensis_SA15
Bborjensis_SA05
Bborjensis_SA16
Bborjensis_SA12
Bborjensis_SA14
Bborjensis_Y05
Bborjensis_SA03
Bborjensis_SA06
Bborjensis_SA11
Bsierrazulensis_SA03
Bsierrazulensis_SA12
Bborjensis_SA08
Bsierrazulensis_SA01
Bsierrazulensis_SA06
Bsierrazulensis_SA11
Bsierrazulensis_SA02
Bsierrazulensis_SA04
Bsierrazulensis_SA08
Bsierrazulensis_SA05
Bsierrazulensis_SA10
Bsierrazulensis_SA07
Bsierrazulensis_SA09
Bxerampelina_O
0.004
B. ceratocarpa
B. huacamayensis
B. sodiroana
B. glabrata
B. borjensis
B. sierrazulensis

Figure 3. Maximum-likelihood phylogenetic tree built using 561 concatenated targeted nuclear loci in IQ-TREE for 116 samples of the six *Burmeitera* study species plus an outgroup (*B. xerampelina*). Brach colors indicate the source population for each sample as shown in the legend and following the colors used in the map from Figure 1. Numbers next to nodes denote bootstrap percentage support values and branch lengths are represented in units of substitutions/site as shown in the scale bar below.

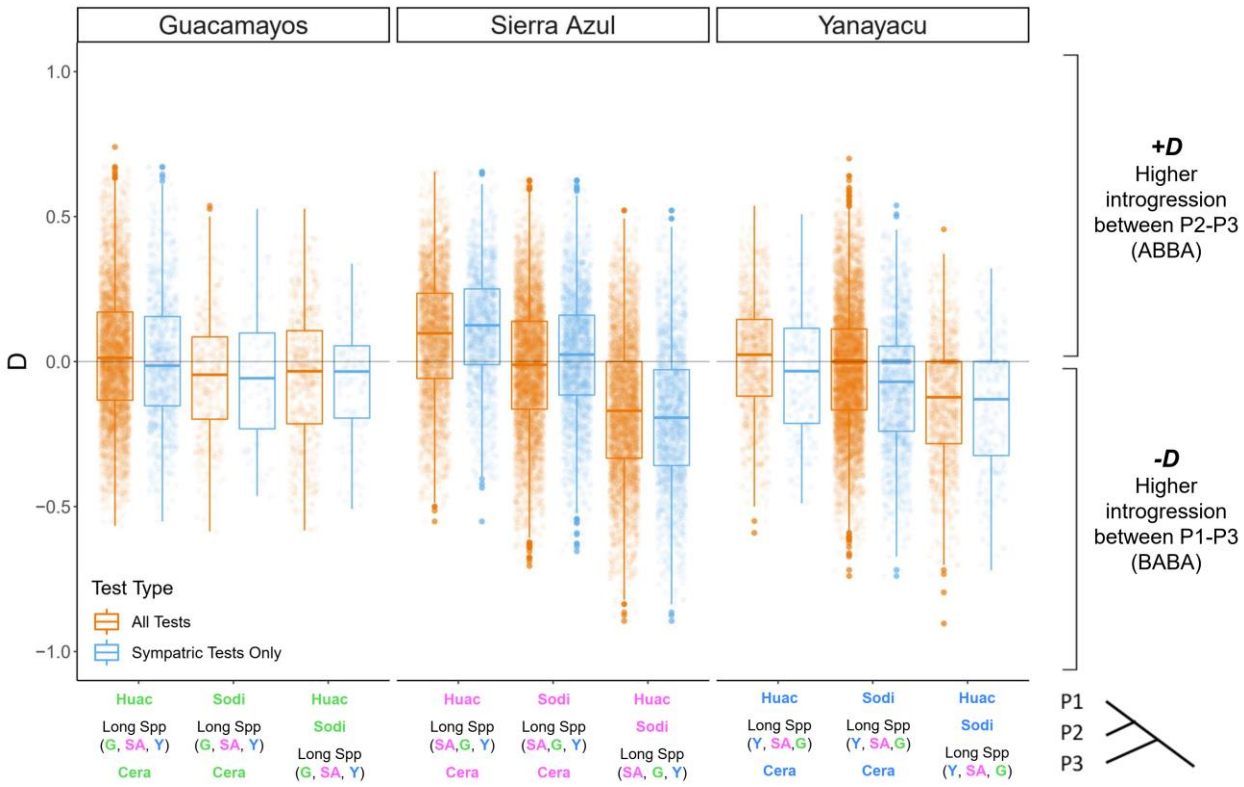

Figure 4. Estimated D-statistic values used to evaluate three different introgression scenarios between short- and long-exserted *Burmeistera* species replicated across the three study sites. Each test included single individual accessions of two sympatric short-exserted species plus a third sample of a long-exserted species all arranged following the species relationships (see Figure 3). Tests were then replicated across all possible individual combinations of the species accessions that fitted the test topology. Shown are separate results from tests that used all samples from long-exserted species across the three study sites and tests that only included accessions from long-exserted species that were sympatric to the short-exserted species used in the test. In all cases, mean D values estimated from all replicates within each test were not significantly different from zero and thus our data does not support any of the introgression scenarios considered. Species abbreviations: Cera: *B. ceratocarpa*; Huac: *B. huacamayensis*; Sodi: *B. sodiroana*; Population abbreviations: G: Guacamayos; SA: Sierra Azul; Y: Yanayacu

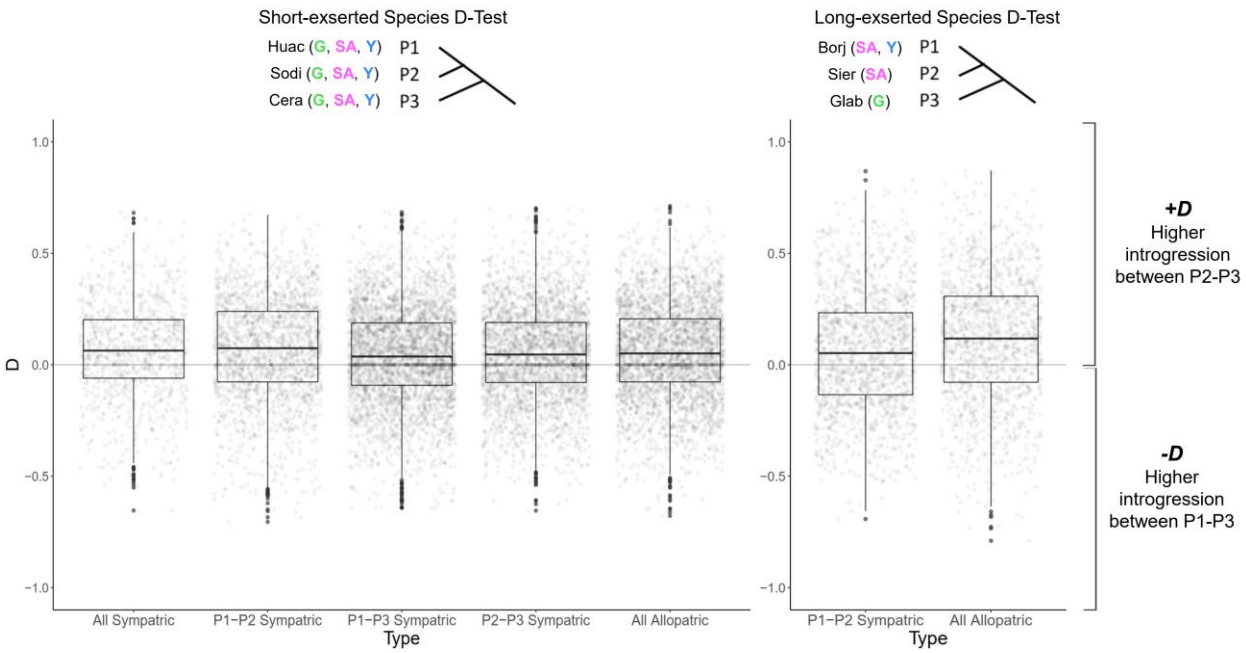

Figure 5. Estimated D-statistic values used to test for introgression between the *Burmeistera* study species with similar exsertion lengths. Each test replicate included one sample from each of the species considered in fixed positions according to their relationships (see Figure 3) and we performed separate tests using different combinations of allopatric and sympatric individuals to evaluate whether introgression signals were stronger between sympatric populations. In all cases, mean D values estimated from all replicates within each test type were not significantly different from zero and thus our data does not support the introgression scenarios considered. Species abbreviations: Borj: *B. borjensis*; Cera: *B. ceratocarpa*; Glab: *B. glabrata*; Huac: *B. huacamayensis*; Sodi: *B. sodiroana*; Sier: *B. sierrazulensis*; Population abbreviations: G: Guacamayos; SA: Sierra Azul; Y: Yanayacu

TABLES

Table 1. Flower type, exsertion length and number of DNA samples for the six bat-pollinated *Burmeistera* study species from three cloud forest locations in Ecuador.

Site	Species	Flower Type	Exsertion Length (mm) Mean ± SD (*N*)	No. DNA samples
Guacamayos	*B. glabrata*	Long-exserted	23.31 ± 1.79 (15)	12
	B. ceratocarpa	Short-exserted	15.68 ± 0.44 (16)	7
	B. huacamayensis	Short-exserted	11.07 ± 0.64 (13)	12
	B. sodiroana	Short-exserted	14.82 ± 0.79 (10)	1
Yanayacu	*B. borjensis*	Long-exserted	24.17 ± 3.26 (18)	11
	B. ceratocarpa	Short-exserted	16.61 ± 0.87 (15)	10
	B. huacamayensis	Short-exserted	11.26 ± 0.71 (15)	2
	B. sodiroana	Short-exserted	15.02 ± 0.59 (15)	11
Sierra Azul	*B. borjensis*	Long-exserted	25.71 ± 1.02 (16)	12
	B. sierrazulensis	Long-exserted	23.29 ± 0.88 (12)	12
	B. ceratocarpa	Short-exserted	17.01 ± 0.72 (15)	8
	B. huacamayensis	Short-exserted	11.73 ± 0.54 (12)	7
	B. sodiroana	Short-exserted	13.44 ± 0.66 (12)	11

Table 2. Results of D-statistic tests to estimate introgressive gene flow between short- and long-exserted *Burmeistera* species from three cloud forest sites in Ecuador. Test Z-scores and P values were calculated from the distribution of D values across all replicates within each test and significance was assessed against the null hypothesis of no introgression (i.e. Mean D equals zero) after adjusting the P values for multiple comparisons using Bonferroni correction. The position of long-exserted species used in each test according to the species relationships (see Figure 3) is indicated by a grey background.

Site	Test	Type	Taxa Included (Population)[1]			No. Replicate Tests	Mean *D*	*D* SD	Z Score	*P* Value
			P1	P2	P3					
Guacamayos	G1	All Long-Exserted Spp	Huac (G)	Glab (G), Borj (SA, Y) & Sier (SA)	Cera (G)	3780	0.03	0.23	-0.109	0.913
		Sympatric Long-Exserted Only	Huac (G)	Glab (G)	Cera (G)	924	0.01	0.22	-0.041	0.967
	G2	All Long-Exserted Spp	Sodi (G)	Glab (G), Borj (SA, Y) & Sier (SA)	Cera (G)	315	-0.04	0.20	0.242	0.809
		Sympatric Long-Exserted Only	Sodi (G)	Glab (G)	Cera (G)	77	-0.05	0.23	0.265	0.791
	G3	All Long-Exserted Spp	Huac (G)	Sodi (G)	Glab (G), Borj (SA, Y) & Sier (SA)	540	-0.05	0.23	0.233	0.816
		Sympatric Long-Exserted Only	Huac (G)	Sodi (G)	Glab (G)	132	-0.06	0.19	0.304	0.761
Sierra Azul	S1	All Long-Exserted Spp	Huac (SA)	Borj (SA, Y), Sier (SA), & Glab (G)	Cera (SA)	2520	0.05	0.22	-0.394	0.694
		Sympatric Long-Exserted Only	Huac (SA)	Borj (SA) & Sier (SA)	Cera (SA)	1288	0.12	0.20	-0.589	0.556
	S2	All Long-Exserted Spp	Sodi (SA)	Borj (SA, Y), Sier (SA), & Glab (G)	Cera (SA)	3960	-0.05	0.23	0.093	0.926
		Sympatric Long-Exserted Only	Sodi (SA)	Borj (SA) & Sier (SA)	Cera (SA)	2024	0.02	0.21	-0.080	0.936
	S3	All Long-Exserted Spp	Huac (SA)	Sodi (SA)	Borj (SA, Y), Sier (SA), & Glab (G)	3465	-0.14	0.23	0.675	0.500
		Sympatric Long-Exserted Only	Huac (SA)	Sodi (SA)	Borj (SA) & Sier (SA)	1771	-0.19	0.24	0.748	0.454
Yanayacu	Y1	All Long-Exserted Spp	Huac (Y)	Borj (Y, SA), Sier (SA), & Glab (G)	Cera (Y)	900	0.03	0.19	-0.050	0.960
		Sympatric Long-Exserted Only	Huac (Y)	Borj (Y)	Cera (Y)	220	-0.04	0.21	0.210	0.834
	Y2	All Long-Exserted Spp	Sodi (Y)	Borj (Y, SA), Sier (SA), & Glab (G)	Cera (Y)	5400	0.00	0.21	0.133	0.894
		Sympatric Long-Exserted Only	Sodi (Y)	Borj (Y)	Cera (Y)	1320	-0.08	0.22	0.405	0.685
	Y3	All Long-Exserted Spp	Huac (Y)	Sodi (Y)	Borj (Y, SA), Sier (SA), & Glab (G)	1080	-0.13	0.20	0.663	0.507
		Sympatric Long-Exserted Only	Huac (Y)	Sodi (Y)	Borj (Y)	264	-0.15	0.22	0.691	0.490

1 Taxon abbreviations: Borj: *B. borjensis*; Cera: *B. ceratocarpa*; Glab: *B. glabrata*; Huac: *B. huacamayensis*; Sodi: *B. sodiroana*; Sier: *B. sierrazulensis*; Population abbreviations: G: Guacamayos; SA: Sierra Azul; Y: Yanayacu

Table 3. Results of D-statistic tests to estimate introgressive gene flow within the short- and long-exserted *Burmeistera* species groups. Tests were devised to include all geographic pairing combinations in order to test whether introgression was stronger between sympatric individuals of the species tested and we included all combinations of individual samples that fitted the test type criteria. Test Z-scores and P values were calculated from the distribution of D values across all replicates within each test and significance was assessed against the null hypothesis of no introgression (i.e. Mean D equals zero) after adjusting the P values for multiple comparisons with Bonferroni correction.

Short-exserted Species Tests

Test Type	Taxa Included			No. Replicate Tests	Mean *D*	*D* SD	Z Score	*P* Value
	P1	P2	P3					
All Sympatric	Huac	Sodi	Cera	940	0.062	0.216	0.286	0.775
P1-P2 Sympatric	Huac	Sodi	Cera	3300	0.057	0.213	0.268	0.775
P1-P3 Sympatric	Huac	Sodi	Cera	1885	0.069	0.234	0.296	0.767
P2-P3 Sympatric	Huac	Sodi	Cera	3575	0.041	0.214	0.192	0.848
All Allopatric	Huac	Sodi	Cera	2900	0.049	0.209	0.235	0.814

Long-exserted Species Tests

Test Type	Taxa Included (Population)[1]			No. Replicate Tests	Mean *D*	*D* SD	Z Score	*P* Value
	P1	P2	P3					
P1-P2 Sympatric	Borj (SA)	Sier (SA)	Glab (G)	1452	0.059	0.265	0.223	0.824
All Allopatric	Borj (Y)	Sier (SA)	Glab (G)	1331	0.114	0.283	0.402	0.688

1 Taxon abbreviations: Borj: *B. borjensis*; Cera: *B. ceratocarpa*; Glab: *B. glabrata*; Huac: *B. huacamayensis*; Sodi: *B. sodiroana*; Sier: *B. sierrazulensis*; Population abbreviations: G: Guacamayos; SA: Sierra Azul; Y: Yanayacu

CHAPTER 4.

Evolution of reproductive isolation during recent and rapid diversification: timecourse of speciation in Neotropical *Burmeistera* (Capanulaceae: Lobelioideae)

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Abstract

The role of pollinator-mediated isolation in angiosperm speciation has received considerable attention since the time of Darwin, but less attention has been devoted to the importance of post-pollination barriers. Reproductive isolation studies in single species pairs only indicate which barriers currently maintain isolation; only clade-level studies of multiple reproductive barriers in a phylogenetic context can shed light on the order of appearance and relative importance of different isolating mechanisms during speciation. The goal of this study was to unravel the timecourse of speciation in the recent radiation of the bat-pollinated plant genus *Burmeistera* (Campanulaceae: Lobeliodideae). We quantified pre-pollination, gametic and postzygotic isolation between 11 different species pairs across a continuum of evolutionary divergence to test the prediction that post-pollination barriers evolve early on, with pre-pollination isolation only arising secondarily in response to reproductive interference upon secondary contact. Overall, we found strong total isolation among the studied pairs accomplished by the combined action of pre-

and post-pollination barriers. Mean reproductive barrier strength was higher for post-pollination barriers compared to pre-pollination ones, yet because of the sequential nature of reproductive isolation both stages had similar relative contributions to total isolation among pairs. All estimates of post-pollination isolation barriers were significantly asymmetric within pairs, suggesting idiosyncratic patterns in how quickly barriers evolve for any given species. We observed positive linear relationships between time since divergence among pairs and the strength of gametic and postzygotic barriers, but no relationship between divergence time and pre-pollination barriers. Lastly, we found a weak but significant negative relationship between divergence time and asymmetry in post-pollination isolation, with lower asymmetry in more distantly related species pairs. Together, our results suggest that post-pollination isolation evolves early on during the speciation process in *Burmeistera*, with pre-pollination isolation being less importantin the initial stages. This study demonstrates that multiple isolating barriers can arise quickly in a rapid radiation. While it is often assumed that pollinator-mediated isolation is critical to driving speciation, our study provides an alternate diversification scenario where post-pollination isolation alone can effectively promote speciation in the absence of obvious pollinator shifts.

Keywords: hybridization, post-pollination barriers, postzygotic isolation, interspecific pollen transfer, pollinator isolation, gene flow

Original Article to be submitted to New Phytologist

The process of speciation is central to evolutionary biology, as it is the path through by which biological diversity arises on the planet. The remarkable evolutionary success of the extant 350,000 flowering plant species is thought to have been driven in no small part by their mutualistic interactions with animal pollinators (Kay & Sargent, 2009; Ollerton *et al.*, 2011; Van der Niet & Johnson, 2012), which provide pollen and thus gene transport between individuals, populations, and even incipient species on already decidedly distinct evolutionary trajectories. Since the time of Darwin is has been recognized that specialization to different pollinators can promote speciation in angiosperms, as shifts between distinct pollinator groups are frequently observed between pairs of closely-related species which limits gene flow between them (Grant, 1949; Harder & Johnson, 2009; Kay & Sargent, 2009). While researchers have long discussed the importance of pollinator-mediated isolating barriers, post-pollination barriers (gametic and postzygotic isolation) have received far less attention. Most reproductive isolation studies in plants have analyzed reproductive barriers between single pairs of species (e.g. Wolf *et al.*, 2001; Ramsey *et al.*, 2003; Kay, 2006; Sobel & Streisfeld, 2014; Karrenberg *et al.*, 2018)); such studies demonstrate how existing isolating barriers maintain current species boundaries, however, they cannot reveal how barriers have arisen over time nor their relative contribution during early versus late stages of speciation. Only clade-level studies of multiple reproductive barriers in a phylogenetic context can shed light on the order of appearance and relative importance of different isolating mechanisms during evolutionary history (Moyle *et al.*, 2004; Kostyun & Moyle, 2017; Christie & Strauss, 2018).

The prevailing pollinator-mediated speciation model first proposed by Grant (1949) has surprisingly rarely been tested in a rigorous, comparative framework. More specifically, most studies contrasting pollination mode with rates of diversification have only assessed whether such an association exists or not, and rarely offer insights into possible explanatory mechanisms. One alternative hypothesis (Armbruster & Muchhala, 2009) has the causality reversed, such that high

species richness drives the evolution of increased pollinator isolation. The proposed mechanism for this reversed causality is that species from highly diverse clades often co-occur yet they are often strongly reproductively isolated, and thus floral trait differences among them will still evolve in sympatry to reduce the costs of interspecific pollen transfer and competition for pollinators (Armbruster & Muchhala, 2009; Grossenbacher & Whittall, 2011; Eaton *et al.*, 2012; Muchhala *et al.*, 2014; Moreira-Hernández & Muchhala, 2019). According to this hypothesis, reproductive character displacement in floral traits mediating pollinator isolation should arise, but only after speciation is already complete and no further gene flow among diverging species is possible because of strong post-pollination isolation by gametic and postzygotic barriers. This idea follows observations that many pollination systems are not as specialized as often assumed and exhibit substantial pollinator sharing, in terms of either visitation patterns (Campbell *et al.*, 1998; Emms & Arnold, 2000; Natalis & Wesselingh, 2012; Randle *et al.*, 2018) or interspecific pollen transfer (Muchhala, 2006; Briscoe Runquist, 2012; Tong & Huang, 2016), casting doubt into whether pre-pollination isolation alone might have driven the initial stages of speciation.

Charting the timecourse of speciation in a clade requires elucidating the relationship between divergence time and the strength of multiple reproductive isolation barriers. This approach uses multiple species pairs of various ages, from recently diverged to long-separated, to compare the relative importance of different isolating barriers and distinguish which are responsible for the initial reduction in gene flow from those that only arose after speciation was complete (Coyne & Orr, 2004). Studies leveraging this approach have indeed found strong correlations between postzygotic barriers and time since divergence for several plant genera (Moyle *et al.*, 2004; Kostyun & Moyle, 2017; Christie & Strauss, 2018). Two of these studies have provided further evidence that pollinator-mediated floral divergence was unrelated with time since divergence and thus likely had a more limited role in promoting evolutionary divergence between species pairs compared to postzygotic barriers (Kostyun & Moyle, 2017; Christie &

Strauss, 2018), in line with the idea that pre-pollination isolation may not in fact play a central role in the initial stages of angiosperm speciation.

The goal of this study was to unravel the timecourse of speciation in the bat-pollinated plant genus *Burmeistera* (Campanulaceae: Lobeliodideae), a recent explosive Andean radiation (~130 spp; <2.5 ma; Figure 1A; Lagomarsino *et al.*, 2016). To accomplish this, we quantified multiple isolation barriers between 11 different species pairs across a continuum of evolutionary divergence. We hypothesize that post-pollination (gametic and postzygotic) barriers have played a critical role during speciation, and that pollinator-mediated (i.e. pre-) pollination isolation has arisen secondarily in response to reproductive interference upon secondary contact (Figure 1B-C). *Burmeistera* flowers deposit pollen in precise areas on the bodies of their bat pollinators determined by different lengths of anther/stigma exsertion (hereafter *exsertion length*; Figure 1A), creating divergence in pollen placement patterns that reduce, but do not completely prevent, interspecific pollen transfer and associated opportunities for genetic exchange. Previous work revealed a pattern of reproductive character displacement in pollen placement among sympatric *Burmeistera*, in that co-occurring species use different portions of bats' bodies to transfer their pollen (Muchhala & Potts 2007). Whether this mechanism of pollinator isolation arose after post-pollination barriers were already in place is currently unknown. However, our most recent studies have shown strong post-pollination isolation between closely related *Burmeistera* species despite the fact that pollinator isolation is incomplete (Muchhala & Potts, 2007; Moreira-Hernández *et al.*, 2019; Chapter 2). If our hypothesis holds, it would provide an alternative to the established pollinator-mediated speciation model revealing how diversification may proceed despite weak pollinator isolation and in the absence of obvious pollinator shifts (Armbruster & Muchhala, 2009; Kay & Sargent, 2009; Ellis & Anderson, 2012; Van der Niet & Johnson, 2012). Therefore, we tested our central hypothesis by completing the following main objectives: 1) obtain quantitative estimates of mean barrier strength at the pre- and post-pollination isolation stages; 2)

determine the absolute and relative contributions of pre- and post-pollination barriers to total reproductive isolation between species pairs; and 3) quantify the relationships between pre- and post-pollination barrier strength with time since divergence using our latest dated *Burmeistera* phylogeny (Bagley *et al.*, 2020).

MATERIALS AND METHODS

Focal taxa and study populations. —*Burmeistera* H. Karst. & Triana (Campanulaceae: Lobelioideae; Figure 1A) is a clade of terrestrial and hemi-epiphytic herbs and shrubs comprising approximately ~130 spp. found at middle and high elevation cloud forests from Guatemala to Northern Peru (Lammers, 2007; Knox *et al.*, 2008; Lagomarsino *et al.*, 2014). The centre of diversity is found in Colombia (~80 spp) and Ecuador (~50 spp), where any given cloud forest location may have 4-6 (sometimes up to 8) sympatric species co-flowering year-round (Muchhala, 2006; Lammers, 2007; Garzón-Venegas & González, 2012; Mashburn, 2019). The zygomorphic flowers are protandrous and possess a staminal column projecting outside of the corolla tube opening at the tip of which the reproductive parts are located (Muchhala, 2006, 2008). Anthesis begins with the male phase; as the corolla tube opens the anthers at tip of the staminal column start releasing copious pollen for the first 24-48 h. After that period, the stigma begins protruding from inside the staminal column pushing off any remaining pollen before expanding to reveal the shiny stigma surface, thus preventing self-pollination (Muchhala, 2006). The female-phase may last for several days but the stigma surface visibly changes from wet, bright, and smooth during the first 48 h to dry, dull, and withered before flowers are eventually shed. *Burmeistera* species are predominantly pollinated by small nectar-feeding bats (Phyllostomidae: Glossophaginae), with hummingbird pollination being restricted to only a handful of species (Muchhala, 2006; Lagomarsino *et al.*, 2017). The fruits are berries which can

be either fleshy or inflated and hollow, and contain hundreds to thousands of small seeds (Lagomarsino *et al.*, 2014; Gamba *et al.*, 2017).

We quantified pre- and post-pollination isolation in 11 *Burmeistera* species pairs through field experiments conducted with multiple populations in different cloud forest locations from Colombia and Ecuador (Table 1). In Colombia, we studied 6 species pairs that naturally co-occur: *B. ceratocarpa-B. sylvicola, B. ceratocarpa-B. succulenta, B. ceratocarpa-B. xerampelina, B. sylvicola-B. succulenta, B. sylvicola-B. xerampelina,* and *B. succulenta-B. xerampelina*. Populations of these species were located on the mountainous slopes northwest of Cali on the Western Colombian Andes in and around the site known as Km 18 on the way to Buenaventura between 1800-2200 masl (3°31'01" N, 76°37'15" W). In Ecuador, we studied the following 5 species pairs: *B. borjensis-B. asclepiadea, B. borjensis-B. glabrata, B. borjensis-B. subcrenata, B. asclepiadea-B. glabrata,* and *B. asclepiadea-B. subcrenata*. Of these, only *B. borjensis-B. asclepiadea* and *B. asclepiadea-B. glabrata* co-occur naturally; the three other pairs all contain species that do not overlap in distribution to the best of our knowledge after several years of fieldwork in Ecuador and a thorough review of herbarium collections (J.I. Moreira-Hernandez & N. Muchhala, personal observation). Study populations of these Ecuadorian species were located between 1400-2200 masl near the towns of Cosanga and Jondachi on the eastern slopes of the Ecuadorian Andes along a 30 km stretch of the E45 road between Quito and Tena (0°37'24" S, 77°50'25" W). In each of the two study areas we searched exhaustively for multiple populations of each species that were separated by at least 10 km to obtain enough focal plants bearing flowers for our hand-pollination experiments as well as enough individuals to serve as pollen donors (see below).

Quantifying pre- and post-pollination isolation. — In *Burmeistera*, previous work suggests that pre-pollination isolation between pairs of species is dictated solely by the degree of overlap in pollen placement on the bat pollinators' bodies (Muchhala & Potts, 2007; Muchhala,

2008). Pollen placement is itself determined by exsertion length and there is a correlation between differences in this trait and the amount of pollen bats transfer between a given species pair (Muchhala & Potts, 2007). Therefore, quantifying pre-pollination isolation can be reduced to this floral measurement which we obtained from flowers in the field and from herbarium specimens (drying and pressing do not change this or other measurements of *Burmeistera* flower morphology; N. Muchhala, personal observation). Exsertion length was measured in mm as the distance separating the tip of the staminal column (taken from the center of anther/stigma) and the constriction of the corolla tube, which is the deepest the snout of bats can probe inside the flower, therefore this distance determines how far back in the head of the bat pollen is deposited during flower visitation. After obtaining mean exsertion length values for all study species, we calculated the absolute difference in this trait between both members of each pair. To calculate an estimate of pre-pollination isolation we used the relationship between exsertion length difference and proportion of conspecific pollen transfer by bats for a pair of *Burmeistera* species, which was determined during controlled experiments by Muchhala and Potts (2007). This relationship follows the formula $y=(ax+1)/(ax+2)$; where y is the proportion of pollen that bats would successfully transfer to conspecific stigmas of the species pair in question (CPT), x is the exsertion length difference between the pair, and a is a constant equal to 0.5985. CPT is therefore constrained between 0 and 1 and it is positively correlated with exsertion length difference; the greater the difference the more conspecific pollen (and thus less heterospecific pollen) is deposited by bats onto stigmas of both species in the pair. Finally, we converted CPT to an index of pre-pollination isolation ($RI_{Pre\text{-}pollination}$) as follows $RI_{Pre\text{-}pollination}=(CPT-0.5)*2$. Thus, when pollen is equally likely to be transferred either conspecifically or heterospecifically (CPT=0.5), there is no isolation ($RI_{Pre\text{-}pollination}=0$), while no heterospecific transfer (CPT=1) corresponds to complete isolation ($RI_{Pre\text{-}pollination}=1$).

To estimate post-pollination isolation, we conducted hand-pollination crosses in the field for each of our 11 species pairs and quantified resulting fruit and seeds produced as well as seed abortion rates (Table 1). For these crosses we selected 15-20 focal plants of each species from different populations around our two main study sites, particularly targeting individuals with many open flowers and buds for the experiments. Other individuals from the same populations were also used opportunistically as pollen donors. For a given species pair, all crosses were performed in both directions (i.e., alternating which species was the pollen donor vs. recipient). We always performed heterospecific pollinations on a given species using pollen from individuals from an allopatric population of the other species member of the pair. We did this to avoid the influence of potential reinforcing selection of reproductive barriers in sympatry (e.g. Kay & Schemske, 2008). In addition, we also performed conspecific pollinations as controls using pollen from sympatric individuals of the same species. This allowed us to quantify post-pollination isolation by comparing the outcome of allopatric heterospecific crosses against sympatric conspecific crosses for each crossing direction within each pair.

The procedure for our hand-pollination crosses in the field was as follows. Every day during fieldwork we visited our study populations to collect pollen from male flowers of the study species and used it to pollinate experimental flowers that had been bagged the day before with bridal veil cloth before they entered the female phase. After removing the bag, we confirmed that stigmas free shiny, bright and free of pollen before they were used for experiments. We applied fresh-collected pollen by gently pressing the stigma against pollen that we carried inside small, labelled paper envelopes, ensuring that all of the stigmatic surface was covered by pollen. In all but three cases, we replicated the above procedure in at least 10 flowers per species for each crossing direction within each pair and for the controls (Table 1). For species that were included in two or more of our species pairs, we avoided performing multiple different crosses within single individual plants whenever possible during the course of fieldwork, and always tried to

randomize the selection of both plants and flowers that were to receive a particular pollen type. After each pollination, we bagged the flowers again to prevent additional pollen deposition by potential visitors and then marked and labeled the flower pedicel and the subjacent branch node with tape. After five weeks we visited our sites to determine fruit fate (matured, aborted, or lost), and collected all mature fruits in 70% alcohol and transported them to the lab to estimate total seed production per fruit and quantify the number of aborted seeds. For each cross, we quantifiedfruit set, seed set, and proportion of seeds aborted. Fruit set was determined as the proportion of hand-pollination crosses that developed into mature fruits with seeds. Similarly, seed set was quantified as the number of mature seeds produced per fruit. For seed abortion rates, we divided the number of aborted seeds by the total sum of aborted and mature seeds produced by that fruit.

We converted these estimates into indexes of reproductive isolation at different post-pollination stages by dividing the outcome of heterospecific crosses (H) by that of conspecific crosses (C) and subtracting this from one (i.e. 1-(H/C); Coyne & Orr, 2004), which yield values between 0 (no isolation) and 1 (complete isolation). We performed these calculations to obtain estimates of post-pollination reproductive isolation at the fruit set ($RI_{FruitSet}$) and seed set ($RI_{SeedSet}$) gametic stages, as well as the early postzygotic seed abortion ($RI_{EarlyPostzygotic}$) stage. We estimated these reproductive isolation values for both directions within each pair and averaged them at each stage to calculate total post-pollination isolation ($RI_{Post\text{-}pollination}$). Given that barrier strength in both crossing directions in each pair could be asymmetric (Moyle *et al.*, 2004; Lowry *et al.*, 2008), we estimated the absolute magnitude of the difference between post-pollination barrier estimates from reciprocal crosses to assess asymmetry across pairs for individual post-pollination barriers. Finally, we followed the methods by Sobel and Chen (2014) to determine for each pair the strength of each barrier along the sequence of reproductive isolation (thus accounting for how much early-acting barriers affect the contribution to isolation by late-acting barriers), total

cumulative isolation granted by all pre- and post-pollination barriers, and both the absolute and relative contributions of each barrier to total isolation.

Divergence time estimation. — To obtain estimates of time since divergence for our 11 species pairs we used the latest dated *Burmeistera* phylogeny based on a phylogenomics dataset of 329 targeted nuclear loci for 125 *Burmeistera* species plus 10 outgroup taxa using methods described by Bagley et al. (2020). We estimated species relationships using a concatened supermatrix and maximum likelihood approach (CAML) in IQ-TREE v2.0.3 (Nguyen *et al.*, 2015), and we calculated best-fit parameters and appropriate models for tree estimation using ModelFinder with percent nodal support obtained from 1000 ultrafast bootstraps pseudoreplicates based on the GTR+ nucleotide substitution model (Kalyaanamoorthy *et al.*, 2017; Hoang *et al.*, 2018). We then converted the resulting CAML phylogeny into a time tree using penalized likelihood (PL; Sanderson, 2002) as implemented by the *chronos* function of the package APE v5.0 (Paradis & Schliep, 2018) in R v3.5.3. (R Development Core Team, 2018). As described in Bagley et al. (2020), we ran the analysis in APE after specifying a relaxed molecular clock using two secondary calibration points based on 95% confidence intervals of molecular divergence times obtained by a previous Bayesian analysis of diversification in *Burmeistera* and related genera (Lagomarsino et al., 2016). We ran PL models over a range of lambda values in APE and identified that the best smoothing parameter corresponded to $\lambda = 0$, meaning that rates of divergence along branches were fully unconstrained in the best supported model. The resulting chronogram (Figure 2) provided robust divergence time estimates for our analyses of reproductive isolation through time across our 11 species pairs.

Statistical analyses. — We used ANOVAs and post-hoc Tukey's HSD to compare the strength of the different isolation barriers across species pairs, including the mean RI strength and the absolute and relative sequential contributions of each barrier to total isolation while accounting for the effect of early-acting barriers on late-acting ones (Sobel & Chen, 2014). To

assess whether the post-pollination barriers were significantly asymmetric across species pairs we used a one-sample *t*-test against the null hypothesis that there were no differences between reciprocal crosses within the same pair. Lastly, we assessed the relationships between pre-pollination, gametic, early postzygotic, and total post-pollination isolation across our species pairs using linear models and linear mixed models. For this, we performed one set of linear models using the mean RI values for each pair (average of both directions), and a second set of linear mixed models using separate RI values for the two different crossing directions within each pair and that also included the maternal species x paternal species interaction as a random effect.

To examine the relationship between barrier strength and time since divergence we used two different approaches. The first consisted of linear regression models using divergence time as the predictor value and the RI values of each barrier (pre-pollination, gametic, early postzygotic, and post-pollination) averaged for each species pair as the response variables. Our second approach used linear mixed models to include the information from both reciprocal crosses performed within each pair by including the maternal species:parental species interaction as a random effect. The response variables in these mixed models were the individual RI values for each reciprocal cross from each pair, thus we only included gametic, early postzygotic, and total post-pollination isolation in this second set of analyses. We predicted that gametic and postzygotic isolation evolved early and rapidly during speciation, so we expected them to correlate strongly with time since divergence. In contrast, we expected pre-pollination isolation to have a non-significant relationship with divergence time, predicting that exsertion length differences do not evolve during speciation, but instead later in response to the particular congeners a given species co-occurs with rather. In addition, we also utilized ordinary linear regressions to explore the relationship between magnitude of asymmetry in barrier strength for each pair and time since divergence. We performed all these analyses in the R programming language (R Development Core Team, 2018).

RESULTS

Variation in pre- and post-pollination barriers across 11 Burmeistera *species pairs.* — We performed a total of 480 hand-pollination crosses in the field including both reciprocal crossing directions for each of our 11 species pairs as well as the controls (Table 1). Combining the results from these crosses together with measurements of exsertion length differences between both species in each pair, we found highly variable estimates of reproductive isolation between pairs across the different stages that we quantified (Table 2). Pairs were isolated by low to high levels of pre-pollination isolation (mean ± sd: $RI_{Pre\text{-}pollination}$ = 0.537 ± 0.269; range: 0.153-0.834). Variation in mean fruit and seed set RI values tended to be similar ($RI_{FruitSet}$: mean ± sd: 0.564 ± 0312; range: -0.200-0.790; $RI_{SeedSet}$: mean ± sd: 0.690 ± 0.184; range: 0.315-0.960), with some reciprocal crosses failing altogether in few cases (Table 2). In two instances (*B. subcrenata*→*B. asclepiadea* and *B. subcrenata*→ *B. borjensis*), slightly more fruits were produced from heterospecific crosses than from conspecific crosses leading to negative RI values, although conspecific fruits outperformed heterospecific ones in the seed set and early postzygotic seed abortion stages (Table 2). Considering gametic isolation as a whole, levels of isolation across pairs were high ($RI_{Gametic}$: mean ± sd: 0.784 ± 0.252; range: 0.152-0.983). At the early postzygotic stage, isolation exhibited more intermediate values ($RI_{EarlyPostzygotic}$: mean ± sd: 0.456 ± 0.203; range: 0.070-0.775) but when combined with gametic isolation the estimated total post-pollination barrier across pairs was high ($RI_{Post\text{-}pollination}$: mean ± sd: 0.869 ± 0.168; range: 0.482-0.992). Together, pre- and post-pollination isolation resulted in near complete isolation across pairs (Total RI: mean ± sd: 0.923 ± 0.108; range: 0.684-0.999).

Mean barrier strength, absolute and relative contributions to total reproductive isolation. — We found significant differences in the strength of reproductive isolation among the barriers we quantified across multiple stages in our species pairs (Table 3; Figure 3). When considering

barriers individually, the mean strength of reproductive isolation did not vary between them (F = 1.69, P= 0.1830; Table 3; Figure 3). However, combining fruit and seed set revealed that gametic isolation was significantly stronger on average than early postzygotic isolation during seed abortion (Tukey HSD, P = 0.0097), and marginally greater than pre-pollination isolation (Tukey HSD, P = 0.0590; F = 5.44, P= 0.0097; Table 3; Figure 3). Likewise, comparing the stages of pre- and post-pollination isolation showed that the latter was significantly stronger among species pairs (F = 12.09, P= 0.0024; Table 3; Figure 3).

In terms of the absolute contribution to total reproductive isolation among species pairs, pre-pollination isolation had an average greater contribution to isolation compared to any of the other individual barriers (Tukey HSD, P < 0.0020 for all pairwise comparisons with other barriers; F = 17.08, P < 0.0001; Table 3). However, this difference disappeared when comparing pre-pollination isolation against gametic (Tukey HSD, P = 0.1630; Table 3), and total post-pollination isolation (Tukey HSD, P = 0.1720; Table 3). Considering the relative contribution to total reproductive isolation instead, pre-pollination isolation once again had a significantly greater mean relative contribution than fruit or seed set and seed abortion (Tukey HSD, P < 0.0005 for all pairwise comparisons with other barriers; F = 16.94, P < 0.0001; Table 3). This was also the case when comparing the mean relative contribution of pre-pollination isolation against combined gametic isolation although the difference was marginal (Tukey HSD, P = 0.0420; F = 17.18, P < 0.0001; Table 3). Lastly, uniting both gametic and early postzygotic into total post-pollination isolation resulted in a similar mean relative contribution to total isolation as that of the pre-pollination barrier (F = 2.52, P = 0.1280; Table 3).

Our results also revealed significant asymmetry in post-pollination barriers across our study species pairs (Table 3). All five stages of post-pollination isolation considered (fruit set, seed set, combined gametic, early postzygotic, and combined total post-pollination) exhibited differences in magnitude depending on the crossing direction (Table 2), and one sample t-tests

indicated that average asymmetry values across pairs were in all cases significantly different from zero (Table 3).

Relationships between the different reproductive isolation stages. — Our ordinary linear models did not find significant pairwise relationships between pre-pollination and gametic, early postzygotic or total post-pollination isolation (pre-pollination vs gametic: $F = 1.33$, $P = 0.2789$; pre-pollination vs early postzygotic: $F = 2.30$, $P = 0.1639$; gametic vs early postzygotic: $F = 0.85$, $P = 0.3793$; pre-pollination vs post-pollination: $F = 1.60$, $P = 0.2371$). However, considering the RI values for both crossing directions of each pair as well as the maternal:parental species interaction in our linear mixed models revealed a marginally significant relationship between the gametic and early-postzygotic barriers ($X^2 = 3.72$; $P = 0.0538$; Figure 4), and a weak significant positive relationship between pre-pollination and early postzygotic isolation ($X^2 = 4.07$; $P = 0.0438$; Figure 4). In contrast, no relationship was observed between the pre-pollination and gametic barriers ($X^2 = 1.50$; $P = 0.2199$), or between pre-pollination and total post-pollination isolation ($X^2 = 1.40$; $P = 0.2367$; Figure 4).

Relation between divergence time, barrier strength and asymmetry. — Our 11 study species pairs encompass a range of evolutionary divergence from ca. 50 k up to 3.0 ma before present (Figure 2; Table 2), allowing us to examine the relationship between divergence time and our metrics of reproductive isolation. We did not observe a significant relationship between pre-pollination isolation and time since divergence for our 11 *Burmeistera* species pairs ($F = 2.40$, $P = 0.1560$; Figure 5A). However, we found positive relationships between time since divergence and gametic, early postzygotic, and total post-pollination isolation (Figure 5). Our linear model including divergence time and gametic isolation revealed a weak positive relationship that approached significance ($F = 4.\ 20$, $P = 0.0759$). However, our mixed linear model that accounted for possible maternal:paternal effects in both reciprocal crosses within each pair found a highly significant positive relationship between gametic isolation and divergence time ($X^2 =$

7.02; $P = 0.0081$; Figure 5B). Similarly, both the ordinary linear model and the mixed model with maternal:paternal random effects found concordant positive relationships between divergence time and early postzygotic isolation (simple linear model: $F = 13.83$, $P = 0.0048$; linear mixed model including both reciprocals and maternal:paternal random effect: $X^2 = 15.28$; $P < 0.0001$; Figure 5C). Both linear model approaches also found a positive association between divergence time and total post-pollination isolation (simple linear model: $F = 8.44$, $P = 0.0174$; linear mixed model including both reciprocals and maternal:paternal random effect: $X^2 = 10.37$; $P < 0.0013$; Figure 5D). Finally, we did not find a relationship between divergence time and asymmetry in gametic ($F = 2.28$, $P = 0.1656$) or early postzygotic isolation ($F = 0.00$, $P = 0.9612$; Figure 6A-B) across our species pairs. However, we observed a negative relationship between divergence time and asymmetry in combined total post-pollination isolation, indicating that crosses between reciprocals were less asymmetric for more distantly related pairs ($F = 5.53$, $P = 0.0432$; Figure 6C).

DISCUSSION

Examining multiple reproductive isolation barriers along a continuum of evolutionary divergence across 11 *Burmeistera* species pairs allowed us to explore how different isolating mechanisms have arisen over time in this rapid plant radiation. We found strong overall total isolation among the studied pairs (Total RI: mean ± sd: 0.923 ± 0.108; range: 0.684-0.999), due to the combined action of pre- and post-pollination barriers. Mean reproductive barrier strength was higher for post-pollination than pre-pollination barriers, yet because of the sequential nature of reproductive isolation both stages had similar relative contributions to the observed total isolation among pairs. We also found that all estimates of post-pollination isolation barriers were significantly asymmetric within pairs, suggesting that barriers isolating different *Burmeistera* species do not evolve at the same rate between them. Finally, we uncovered linear positive

relationships between post-pollination barriers and time since divergence among pairs, and no such relationship for pre-pollination barriers. In addition, we observed an unexpected negative relationship between divergence time and asymmetry in total post-pollination isolation, with lower asymmetry in more distantly related species pairs. Together, these results suggest that gametic and postzygotic barriers conferring strong post-pollination isolation have been very important to prevent gene flow and promote divergence during the diversification of *Burmeistera*, and that current floral differences in exsertion length causing pre-pollination isolation have evolved more recently.

Strong pre- and post-pollination isolation in Burmeistera. — Previous work with *Burmeistera* has established that pre-pollination isolation via exsertion length differences is important to minimize interspecific pollen transfer between sympatric species (Muchhala & Potts, 2007; Muchhala, 2008) and we indeed observed a range of pre-pollination isolation values among our species pairs. However, a formal quantification of post-pollination isolation mechanisms in *Burmeistera* had not been done before this study. We unveiled previously unknown variation in reproductive isolation at multiple post-pollination stages across our study species pairs that resulted in strong isolation between them following heterospecific pollinations. Thus, *Burmeistera* species exhibit significant barriers preventing gene flow which have the potential to successfully prevent the formation of hybrids despite the frequent interspecific pollen transfer that has been observed between co-occurring species in natural conditions (Muchhala, 2006; Chapter 2; Chapter 3). Moreover, our measurement of early seed abortion is only a tiny sliver of potential postzygotic isolation that might manifest across multiple later stages of hybrid development (e.g. seed germination, seedling growth, F1 vigor, F1 fertility, etc). Considering the combined action of both pre- and post-pollination isolation, we can conclude that our study species pairs appear to be almost completely isolated which is remarkable given the recent diversification of the group (<2.5 mya; Lagomarsino *et al.*, 2016).

One of the key patterns from our results is the fact that the high variation in barrier strength and in the absolute and relative contributions of different isolating barriers among our species pairs suggest that no single predominant isolation mechanism drives reproductive isolation in *Burmeistera*. Rather, different pairs are idiosyncratically isolated through the action of barriers at slightly different stages and with varying magnitudes. This pattern has also been observed in other clade-level studies of reproductive isolation (Moyle *et al.*, 2004; Kostyun & Moyle, 2017; Christie & Strauss, 2018).

We also found a marginally significant relationship between gametic isolation and early postzygotic isolation. Most pairs examined had high gametic isolation values for both crossing reciprocals but three of them (all amongst the most recently diverged) had highly asymmetric RI values for this barrier. These three pairs (*B. asclepiadea-B. subcrenata*, *B. borjensis-B. subcrenata*, and *B. ceratocarpa-B. sylvicola*) also had comparatively weaker early postzygotic isolation and for two of them this stage was also asymmetric in the same direction than the gametic barrier. The observed association between gametic and early postzygotic isolation probably reflects the developmental link between fruit and seed production and hybrid seed abortion. For example, if certain heterospecific crosses induce high enough seed abortion to trigger abortion of the whole fruit the outcome would look as if there were a positive association between gametic and early postzygotic isolation (Christie & Strauss, 2018; Coughlan *et al.*, 2020). However, many crosses with low hybrid seed abortion still resulted in high fruit and seed set failure, thus indicating that developmental effects alone probably do not explain the weak relationship between gametic and early postzygotic isolation that we observed.

Our results also showed that the post-pollination isolation barriers we quantified in *Burmeistera* were significantly asymmetric across pairs. This result is also concordant with broader patterns found in the literature; in fact, asymmetry in reproductive isolation seems to be the norm across angiosperms (Tiffin *et al.*, 2001; Lowry *et al.*, 2008; Moreira-Hernández &

Muchhala, 2019). Post-pollination barriers (both gametic and postzygotic) are often found to be highly asymmetrical, up to 3 times more so than pre-pollination barriers (Lowry *et al.*, 2008), and we found that indeed the gametic isolation stage had the greatest levels of asymmetry between our study species pairs. Yet the fact that we observed these asymmetries in a recent plant radiation like *Burmeistera* shows that differences in rates of barrier evolution can arise quickly during diversification. That strong isolation can be generated by the action of multiple reproductive barriers operating at different stages and even asymmetrically within the same species pair, provides a rich set of possibilities for speciation to have occurred rapidly through the rapid diversification of *Burmeistera*.

Timecourse of speciation in the recent radiation of Burmeistera. — Reconstructing the order of appearance of different reproductive isolation barriers along a continuum of evolutionary divergence is key to understand the speciation process. Our data on reproductive isolation across 11 *Burmeistera* species pairs combined with robust divergence time estimates from a well-resolved dated phylogeny allowed us to unravel the evolution of reproductive isolation in this rapid radiation. Our results support our hypothesis that gametic and postzygotic barriers have followed a positive linear relationship with time since divergence, thus highlighting the crucial role post-pollination mechanisms play in the initial stages of speciation (Lowry *et al.*, 2008; Kostyun & Moyle, 2017). Conversely, the strength of current pre-pollination isolation was unrelated to divergence time which suggests that this barrier has not been the main factor limiting historic gene flow among pairs of species. As the rapid radiation of *Burmeistera* has occurred without obvious shifts in pollinators conferring more significant levels of pre-pollination isolation, is it thus clear that speciation in the genus has followed an alternative path to that from the predominant view of pollinator-mediated diversification.

The expectation that the strength of post-pollination (or postmating in animals) reproductive isolation barriers might be correlated to time since divergence comes from multiple

studies that have shown this to be the case in multiple different taxa (Gardner & Macnair, 2000; Coyne & Orr, 2004; Moyle *et al.*, 2004; Coughlan & Matute, 2020). Postzygotic isolation is the clearest example: the gradual accumulation of intrinsic genetic incompatibilities is expected to increase linearly with time since divergence thus reducing the likelihood of generating fit hybrid progeny from crosses between distantly related species pairs (Kostyun & Moyle, 2017; Christie & Strauss, 2018; Coughlan & Matute, 2020). We observed this pattern in our *Burmeistera* species pairs, with seed abortion being higher in crosses between the most distantly related species pairs. Interestingly, our data also suggest a similar positive association between gametic isolation and divergence time. Of the reproductive barriers we considered gametic isolation had the greatest asymmetry, which was more pronounced in the recently diverged species pairs. In some of these cases gametic isolation was comparatively weaker in one of the reciprocals where the maternal species in those crosses still produced some hybrid fruits and seeds whereas crosses in the oppositive direction were less successful and produced RI values that were like those observed amongst the most distantly related species pairs. The strong asymmetries we observed across the gametic barriers also suggest that the evolution of barriers operating at this stage is not very predictable across pairs.

We also observed an unexpected relationship between divergence time and asymmetry of total pre-pollination isolation. This relationship was weakly significant ($p = 0.0432$) and thus we interpret it with caution. On one hand, gametic barriers preventing maladaptive hybridization are expected to be highly asymmetric when the relative costs of intrinsic postzygotic incompatibilities differ significantly between a pair of diverging species (Lowry *et al.*, 2008; Christie & Strauss, 2018). Even though postzygotic isolation can be highly asymmetric during early divergence, its overall magnitude is still expected to increase with time as we observed in *Burmeistera*. Beyond a certain threshold, postzygotic isolation should be strong enough in both crossing directions that natural selection should eliminate any asymmetry in gametic barriers

(which by then should be strong both ways; Lowry *et al.*, 2008; Coughlan *et al.*, 2020), leading to near complete post-pollination isolation that is little asymmetric between the species pair as we observed. Whether this pattern also occurs in other taxa is intriguing and certainly deserves further study.

Conclusion. — Our study demonstrates that multiple barriers conferring reproductive isolation can arise quickly in a rapid radiation and provides an alternate diversification scenario where post-pollination isolation can effectively promote speciation in the absence of obvious pollinator shifts. Much attention has been paid to how specialization to different pollinator species contributes to diversification by promoting reproductive isolation. However, less attention has been devoted to how interactions mediated by the very pollen pollinators carry may contribute to flower evolution, genetic exchange, and reproductive isolation during speciation. We hope this contribution helps alleviate this oversight, by showing how the extraordinary radiation of *Burmeistera* might have taken place while faithfully upholding their close partnership with their furry nectar-seeking bat pollinator friends.

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FIGURES

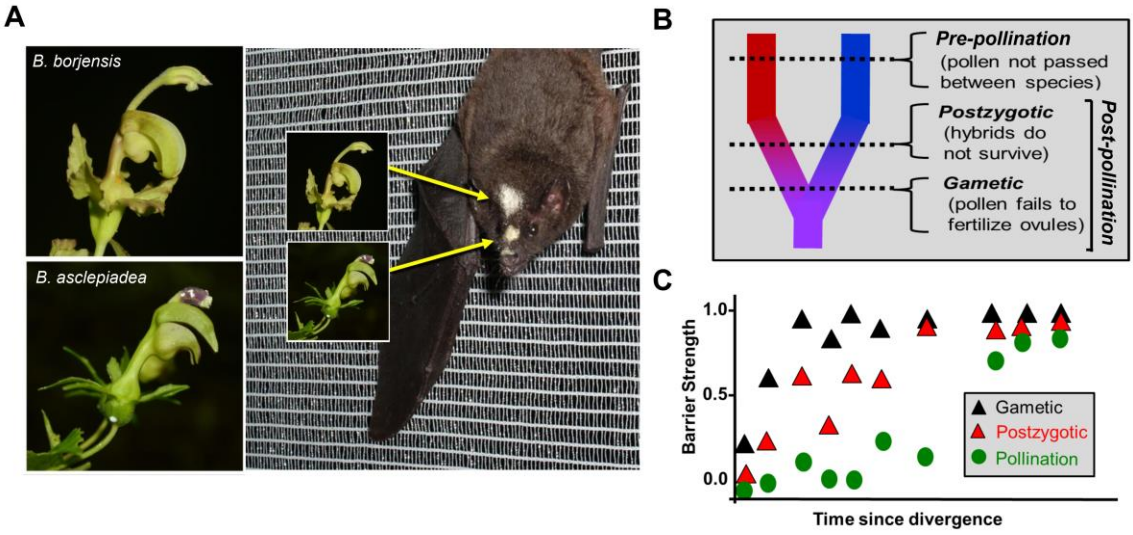
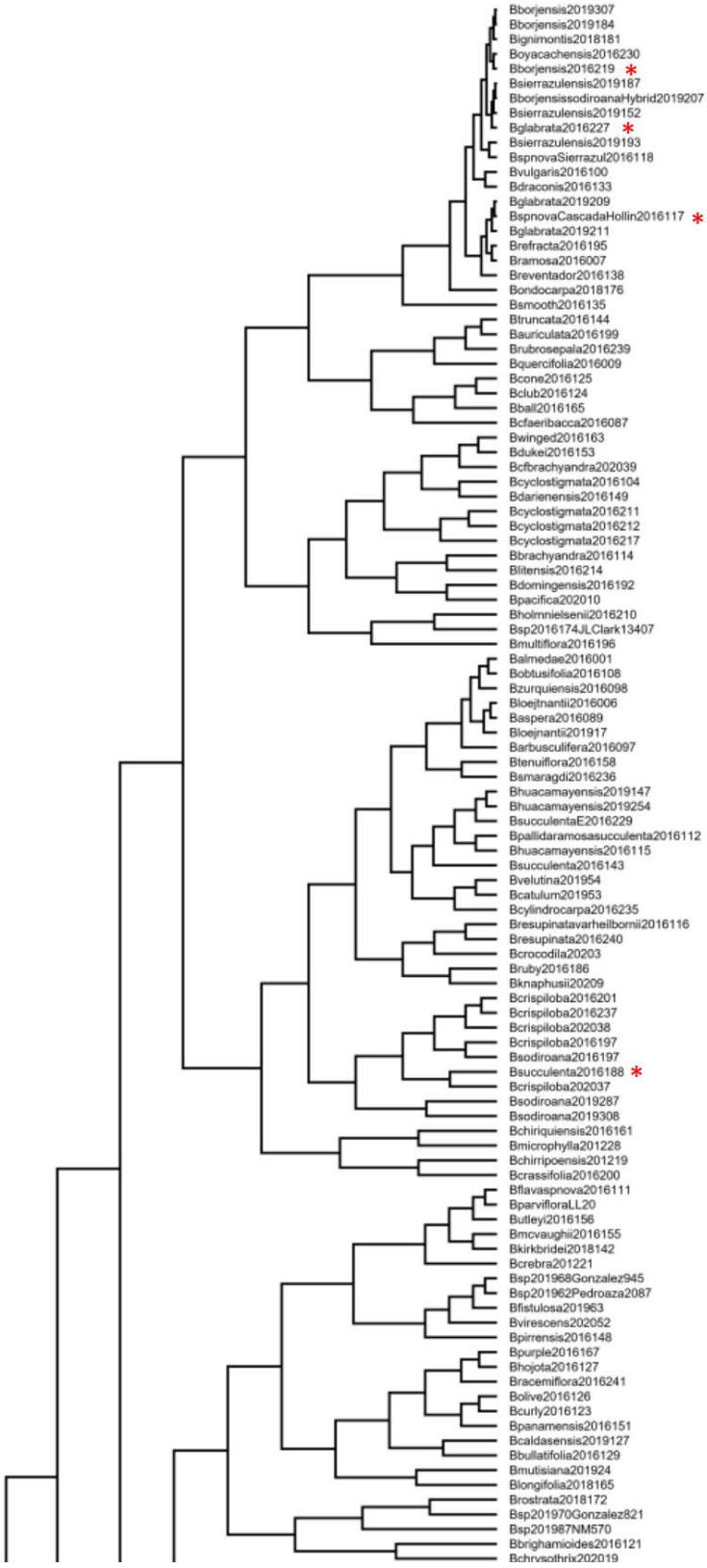


Figure 1. Hypothesized pattern for the evolution of reproductive isolation in bat-pollinated *Burmeistera* and similar recent angiosperm radiations. **A)** Pre-pollination isolation between sympatric *Burmeistera* is conferred by species differences in the length of the anther/stigma exsertion outside of the corolla tube which results in different pollen placement on the bodies of their bat pollinators thus minimizing interspecific pollen transfer (Muchhala & Potts, 2007). However, the importance of post-pollination isolation and the order of appearance of different isolating barriers during the diversification of the group remain unexplored. **B)** We propose that after initial geographic separation, pairs of incipient species evolve gametic and postzygotic barriers during early divergence, followed by pre-pollination isolation later after secondary

contact. **C)** Expected time course of speciation for ten species pairs; symbols show barrier strength for that pair, and the x-axis gives relative time since divergence estimated from a phylogeny. As in **B**, gametic and postzygotic barriers appear first, followed by pre-pollination isolation.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Bglabrata2016227 *
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Bclub2016124
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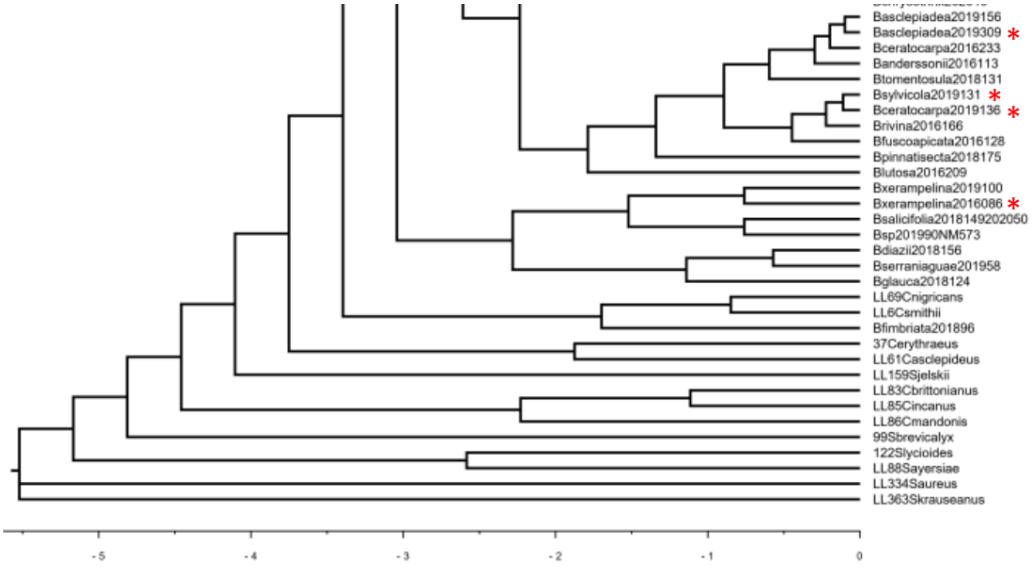

Figure 2. Latest *Burmeistera* time tree estimated using maximum likelihood on a concatenated supermatrix of 329 targeted nuclear loci for 125 *Burmeistera* species and 10 outgroups (Bagley *et al.*, 2020; Bagley *et al.*, in prep.). Tips corresponding to accessions of the study species for which we obtained divergence time estimates are shown with red asterisks.

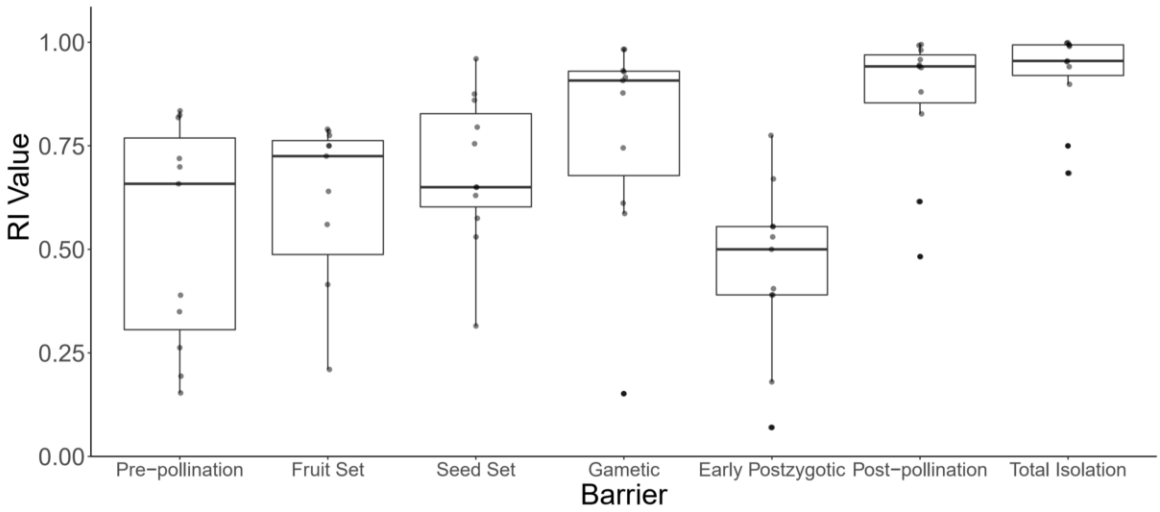

Figure 3. Variation in mean relative strength of pre- and post-pollination isolation barriers quantified for 11 *Burmeistera* species pairs.

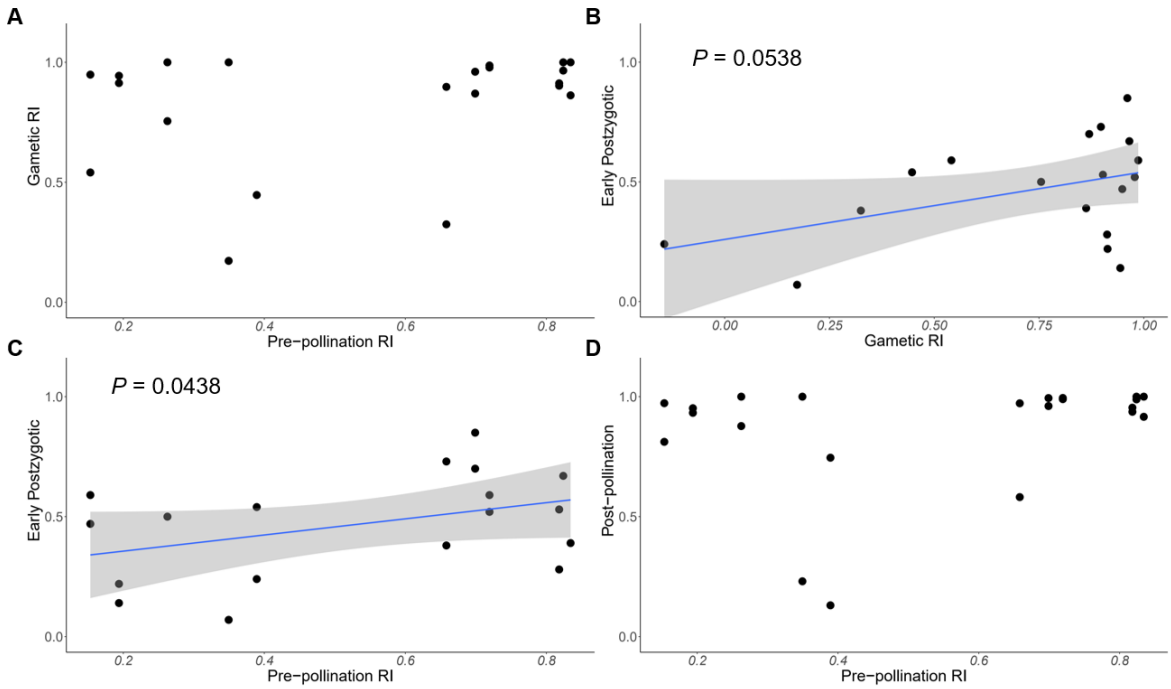

Figure 4. Relationships between different stages of reproductive isolation quantified across 11 *Burmeistera* species pairs. Data shows results from the outcomes of both reciprocal crosses within each pair (N = 22). Significant relationships are shown by blue lines with 95% confidence intervals and associated P values as estimated from linear models that included maternal:paternal species interactions as a random effect.

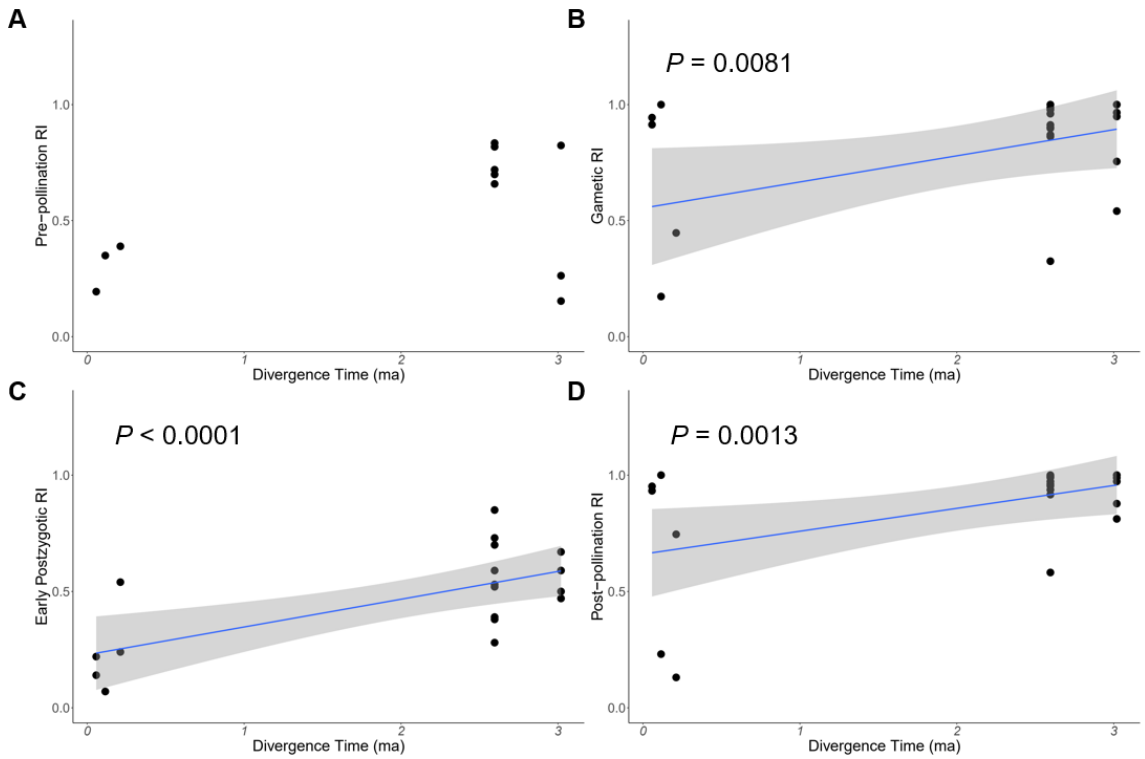

Figure 5. Relationships between divergence time and reproductive barrier strength across pre-pollination (**A**), gametic (**B**), early postzygotic (**C**), and combined post-pollination isolation (**D**) for 11 *Burmeistera* species pairs. Data from **A** reflects pre-pollination isolation from exsertion length differences between both species in each pair (N = 11), while **B**, **C**, and **D** show results from the outcomes of both reciprocal hand-pollination crosses within each pair (N = 22). Significant relationships are shown by blue lines with 95% confidence intervals with associated P values from linear models that included maternal:paternal species interactions as a random effect.

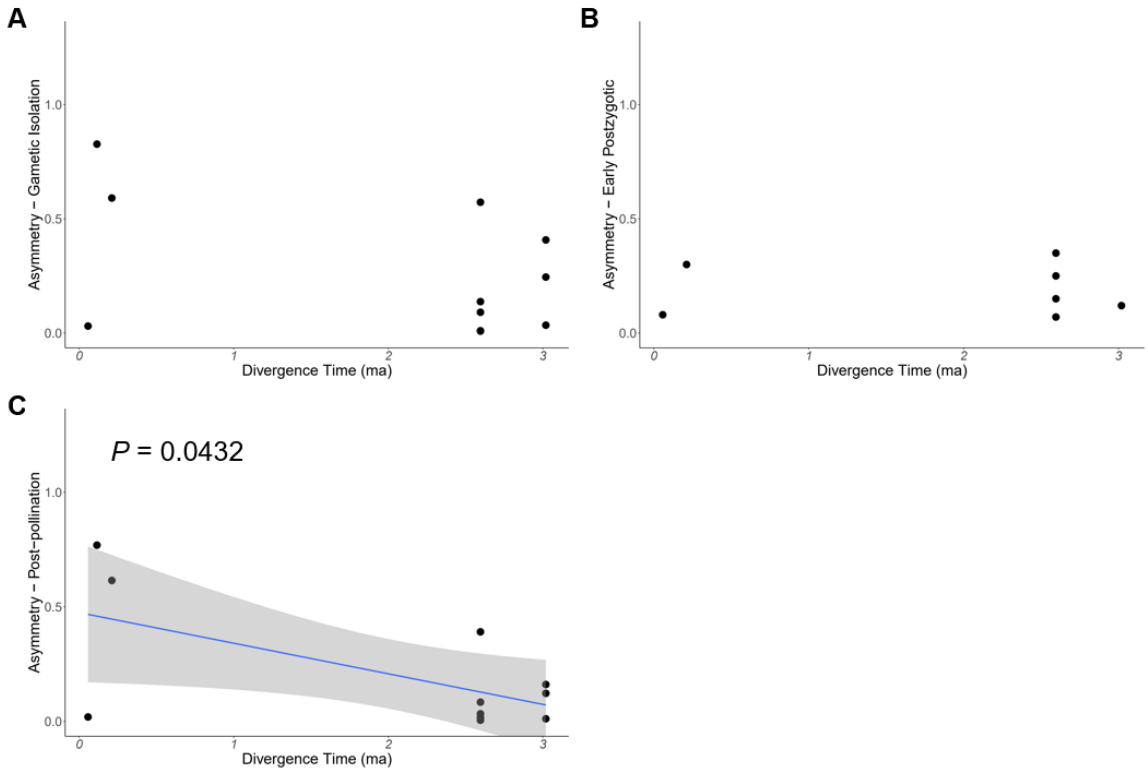

Figure 6. Relationships between divergence time and asymmetry in gametic (**A**), early postzygotic (**B**), and combined post-pollination isolation (**C**) across 11 *Burmeistera* species pairs. Asymmetry was calculated as the absolute magnitude of the difference between the RI values estimated for both reciprocal hand-pollination crosses within each pair (N = 11). Significant relationships are shown by blue lines with 95% confidence intervals and associated P values.

TABLES

Table 1. Divergence time, anther/stigma exsertion length differences, and number of hand-pollination crosses performed in 11 species pairs of bat-pollinated *Burmeistera* (Campanulaceae: Lobeliodeae) from Colombia and Ecuador.

Country	Pair No.	Species Pair		Divergence	Exsertion Length	Crosses 1→2		Crosses 2→1	
		Species 1	Species 2	Time (ma)	Difference (mm)	*N*	Control	*N*	Control
Colombia	1	*B. ceratocarpa*	*B. silvicola*	0.115	1.8	9	12	10	11
	2	*B. ceratocarpa*	*B. succulenta*	2.596	16.8	11	18	12	11
	3	*B. ceratocarpa*	*B. xerampelina*	3.019	1.2	12	13	9	11
	4	*B. silvicola*	*B. succulenta*	2.596	15.0	12	18	12	12
	5	*B. silvicola*	*B. xerampelina*	3.019	0.6	10	13	11	12
	6	*B. succulenta*	*B. xerampelina*	3.019	15.6	7	13	11	18
Ecuador	7	*B. borjensis*	*B. asclepiadea*	2.596	8.6	29	48	37	49
	8	*B. borjensis*	*B. glabrata*	0.058	0.8	13	18	23	49
	9	*B. borjensis*	*B. subcrenata*	0.212	2.1	14	8	11	49
	10	*B. asclepiadea*	*B. glabrata*	2.596	7.8	10	18	13	48
	11	*B. asclepiadea*	*B. subcrenata*	2.596	6.4	16	8	11	48

Table 2. Quantitative estimates of pre- and post-pollination reproductive isolation across 11 species pairs of bat-pollinated *Burmeistera* (Campanulaceae: Lobeliodeae). Species pairs are listed by decreasing divergence time (*i.e.* towards present time).

Pair	Crossing Directions	Divergence Time (ma)	Pre-pollination	Post-pollination														Total RI
			$RI_{Pre\text{-}pollination}$	Gametic									Early Postzygotic			$RI_{Post\text{-}pollination}$			
				$RI_{FruitSet}$			$RI_{SeedSet}$			Total $RI_{Gametic}$			$RI_{EarlyPostzygotic}$						
				Value	Mean	Asymmetry	Value	Mean	Asymmetry	Value	Mean	Asymmetry	Value	Mean	Asymmetry	Value	Mean	Asymmetry	
1	Cera-Xera Xera-Cera	3.019	0.263	1.000 0.500	0.750	0.500	1.000 0.510	0.755	0.490	1.000 0.755	0.878	0.245	- 0.500	0.500	-	1.000 0.878	0.939	0.123	0.955
2	Sylv-Xera Xera-Sylv	3.019	0.153	0.730 0.100	0.415	0.630	0.810 0.490	0.650	0.320	0.949 0.541	0.745	0.408	0.470 0.590	0.530	0.120	0.973 0.812	0.880	0.161	0.898
3	Succ-Xera Xera-Succ	3.019	0.824	1.000 0.570	0.785	0.080	1.000 0.920	0.960	0.080	1.000 0.966	0.983	0.034	- 0.670	0.670	-	1.000 0.989	0.994	0.011	0.999
4	Cera-Succ Succ-Cera	2.596	0.834	0.450 1.000	0.725	0.550	0.750 1.000	0.875	0.250	0.863 1.000	0.931	0.138	0.390 -	0.390	-	0.916 1.000	0.958	0.084	0.993
5	Sylv-Succ Succ-Sylv	2.596	0.818	0.750 0.750	0.750	0.000	0.650 0.610	0.630	0.040	0.913 0.903	0.908	0.010	0.280 0.530	0.405	0.250	0.937 0.954	0.945	0.017	0.990
6	Borj-Ascl Ascl-Borj	2.596	0.719	0.670 0.910	0.790	0.240	0.960 0.760	0.860	0.200	0.987 0.978	0.983	0.008	0.590 0.520	0.555	0.070	0.995 0.990	0.992	0.005	0.998
7	Ascl-Glab Glab-Ascl	2.596	0.699	0.690 0.860	0.775	0.170	0.580 0.720	0.650	0.140	0.870 0.961	0.915	0.091	0.700 0.850	0.775	0.150	0.961 0.994	0.981	0.033	0.994
8	Ascl-Subc Subc-Ascl	2.596	0.658	0.670 -0.250	0.210	0.920	0.690 0.460	0.575	0.230	0.898 0.325	0.611	0.573	0.730 0.380	0.555	0.350	0.972 0.582	0.827	0.391	0.941
9	Borj-Subc Subc-Borj	0.212	0.389	0.030 -0.430	-0.200	0.460	0.430 0.200	0.315	0.230	0.447 -0.144	0.152	0.591	0.540 0.240	0.390	0.300	0.746 0.131	0.482	0.615	0.684
10	Cera-Sylv Sylv-Cera	0.115	0.349	0.120 1.000	0.560	0.880	0.060 1.000	0.530	0.940	0.173 1.000	0.586	0.827	0.070 -	0.070	-	0.231 1.000	0.615	0.769	0.750
11	Borj-Glab Glab-Borj	0.058	0.194	0.600 0.680	0.640	0.080	0.860 0.730	0.795	0.130	0.944 0.914	0.929	0.030	0.140 0.220	0.180	0.080	0.952 0.933	0.942	0.019	0.953

Table 3. Mean RI values, mean cumulative absolute and mean relative contribution to total RI, and mean asymmetry of our estimates of pre- and pollination reproductive isolation barriers across 11 species pairs of bat-pollinated *Burmeistera* (Campanulaceae: Lobeliodeae).

Metric of Reproductive Isolation	Pre-pollination	Post-pollination					Total Isolation
		Gametic			Early Postzygotic	Total Post-pollination	
		Fruit Set	Seed Set	Total Gametic			
Mean RI	0.537^{a}	0.564^{a}	0.690^{a}	0.784^{b*}	0.456^{a}	0.869^{c}	0.923
Mean absolute contribution to total RI	0.537^{*a}	0.235^{b}	0.106^{b}	0.341^{a}	0.042^{b}	0.384^{a}	1.000
Mean relative contribution to total RI	0.586^{a}	0.230^{b}	0.126^{b}	0.356^{b}	0.057^{c}	0.414^{a}	1.000
Mean asymmetry (post-pollination only)		0.410^{*}	0.277^{*}	0.269^{*}	1.320^{*}	0.203^{*}	

For mean RI, and absolute and relative contribution to total RI, small lowercase letters indicate significant pairwise differences between barriers at $P < 0.05$ in all cases except one where the difference was marginal ($P = 0.059$; indicated by b*). For mean asymmetry, small asterisks indicate that significant barrier asymmetry was supported by one sample t-tests against the null hypothesis of no asymmetry (*i.e.* mean asymmetry equal to zero).