A gradient of pollination specialization in three species of Bolivian *Centropogon*

Laura P. Lagomarsino[1,2,3] (iD) and Nathan Muchhala[1] (iD)

PREMISE: Closely related plant species with overlapping ranges often experience competition for pollination services. Such competition can select for divergence in floral traits that attract pollinators or determine pollen placement. While most species in *Centropogon* (Campanulaceae: Lobelioideae) have flowers that suggest adaptation to bat or hummingbird pollination, actual pollinators are rarely documented, and a few species have a mix of traits from both pollination syndromes. We studied the pollination biology of a "mixed-syndrome" species and its co-occurring congeners to examine the relationship between floral traits and visitation patterns for *Centropogon*.

METHODS: Fieldwork at two sites in Bolivian cloud forests involved filming floral visitors, quantifying pollen transfer, and measuring floral traits. Stamen exsertion, which determines pollen placement, was measured from herbarium specimens across the geographic range of these species to test for character displacement.

RESULTS: Results show a generalization gradient, from primarily bat pollination in white-flowered *Centropogon incanus*, to bat pollination with secondary hummingbird pollination in the cream-flowered *C. brittonianus*, to equal reliance on both pollinators in the red-flowered, mixed-syndrome *C. mandonis*. Pollen transfer between these species is further reduced by differences in stamen exsertion that are accentuated in zones of sympatry, a pattern consistent with character displacement.

CONCLUSIONS: Our results demonstrate that key differences in floral color and shape mediate a gradient of specialization in Bolivian *Centropogon*. Interspecific pollen transfer is further reduced by potential character displacement of a key trait. Broadly, our results have implications for understanding the hyper-diversity of Andean cloud forests, in which multiple species of the same genus frequently co-occur.

KEY WORDS bat pollination; centropogonid clade; *Centropogon*; character displacement; ecological specialization; hummingbird pollination; Lobelioideae; Neotropics; plant–animal interactions; pollination syndromes.

The cloud forests of the tropical Andes are one of the most biodiverse regions of the world (Myers et al., 2000). These species-rich communities often include multiple members of large plant genera, creating the potential for strong competition for pollination services among close relatives (Karron, 1987; Mitchell et al., 2009). For these species, minimizing interspecific pollen transfer by partitioning pollinator resources may be crucial to reproductive success (Rathcke, 1983; Waser, 1983; Morales and Traveset, 2008). Effective partitioning reduces the chance of pollen loss to foreign flowers and stigmatic clogging by foreign pollen, and may reduce gene flow in the face of otherwise incomplete reproductive isolation among closely related species. Floral isolation—the interaction between floral traits and pollinator behavior that reduces interspecific pollen transfer (Grant, 1949, 1994)—can be accomplished in multiple ways. For example, precise pollen deposition on distinct parts of a pollinator's body can allow multiple plant species to share the same pollinator (Armbruster et al., 1994). Alternatively, co-occurring plant species may rely on entirely different pollinators. These outcomes can be accomplished via the divergent evolution of traits related to the attraction or prevention of different types of pollinator or those related to pollen placement (Johnson et al., 2006; Raguso, 2008; Clark et al., 2015). Such evolution of floral isolation in response to competition for pollination is a form of reproductive character displacement, and in certain cases may represent reinforcement if it

evolves between closely related species in response to hybridization or introgression that results in less fit phenotypes (Armbruster and Muchhala, 2009; Kay and Sargent, 2009).

The existence of suites of floral characteristics associated with the attraction and utilization of a specific functional group of pollinators (i.e., pollination syndromes; Fenster et al., 2004) demonstrates the outcome of similar selective regimes across distantly related taxa. For example, brightly colored, narrowly tubular flowers are well documented to be effectively and reliably pollinated by hummingbirds (Fenster, 1991; Temeles and Kress, 2003; Martén Rodríguez, 2008; Muchhala et al., 2014), while the same is true of strongly scented wide flowers and pollination by bats (Muchhala, 2003; Martén Rodríguez, 2008; Fleming et al., 2009). While pollination syndromes are often thought to reflect selection by the most effective pollinators of a given plant species (Stebbins, 1970), there is a substantial degree of variation of specialization even within syndromes (Johnson and Steiner, 2000; Mayfield, 2001; Ollerton et al., 2009, 2015). Pollination specialization occurs on a gradient, from extreme generalization, where many species of pollinators across multiple functional groups visit a flower (as seen in prairie Asteraceae; Wagenius and Lyon, 2010), to extreme specialization, where only one species of pollinator visits a flower (as in the pheromone producing, sexually deceptive *Ophrys* orchids; Scopece et al., 2010). Specificity in pollination relationships is determined by a combination of floral morphology, non-morphological traits that provide cues to flower visitors (e.g., scent and color), and flowering phenology and is likely influenced by the number of co-occurring species and by environmental factors such as seasonality (Johnson and Steiner, 2000).

Support for pollination syndromes within the centropogonid clade, a group of ~550 species with its highest species richness in the Andean mountains, was recently documented (Lagomarsino et al., 2017). Species with brightly colored flowers, predicted to be adapted to pollination by hummingbirds, were demonstrated to fall into a separate region of floral morphospace than dull-colored flowers, predicted to be adapted to bat pollination. Among other key traits, bat-adapted flowers tend to have shorter corolla tubes, wider corolla openings, and larger anthers than hummingbird-adapted flowers. These results were corroborated by a linear discriminant analysis, in which floral traits from a handful of species with known pollinator relationships predicted the pollinators of species without known pollinators. While there was broad overlap between these two methods, there were several species for which the pollinator could not be easily predicted because they displayed a combination of traits associated with either bats or hummingbirds. A major goal of the present study was to compare the pollination biology of one of these mixed-syndrome species, the red-flowered *Centropogon mandonis* (Fig. 1A), to two co-occurring *Centropogon* species predicted to be adapted to bat pollination (Fig. 1B, C). Via this comparison, we hope to gain further insight into the evolution of pollination syndromes and floral specialization in this group.

Our three focal species (*C. brittonianus*, *C. incanus*, and *C. mandonis*) belong to the peruvianid subclade (Lagomarsino et al., 2014), a group of ~25 species found in humid montane forests in the central Andes (i.e., of Peru and Bolivia). This clade is predicted to be ancestrally adapted to bat pollination (Lagomarsino et al., 2017), and most species produce wide, cream-colored or white flowers that emit a strong, sulfurous odor. However, a minority of peruvianid species, including *C. mandonis*, have flowers that are brightly colored (i.e., red or pink) and do not have a strong odor. In the present study, which is one of the first pollination biology studies conducted in Bolivia (but see Wester and Clasen-Bockhoff, 2006; Döll et al., 2007; Chumacero de

Schawe et al., 2016) and the first for peruvianid *Centropogon* species, we aimed to determine the extent to which these species share pollinators, as well as the potential role that floral traits play in determining effective pollinators. We further hypothesized that interspecific differences in stamen exsertion length—a trait known to mediate precise pollen placement in close relatives of our focal taxa (Muchhala and Potts, 2007; Muchhala, 2008)—reduce interspecific pollen transfer in this system. We tested this by assessing whether differences are greater in regions of sympatry than in regions of allopatry, a pattern consistent with character displacement. In assessing these aspects of the ecology of our focal species, we also aimed to explore the boundaries between bat and hummingbird pollination syndromes more generally.

MATERIALS AND METHODS

Study system and field sites

Our work focused on three species of *Centropogon*: *C. brittonianus*, *C. incanus*, and *C. mandonis*. These are known to be very closely related—perhaps even each other's closest relatives (Lagomarsino et al., 2014). Whether there is a history of introgression between these taxa has yet to be explored, though the distinct morphology of each species suggests that they are not solely of hybrid origin. Flower color varies between them: *C. incanus* has white flowers, *C. brittonianus* has cream-colored flowers that are usually streaked with maroonish red, and *C. mandonis* has deep red flowers (Fig. 1). Flowers of these species, like all Lobelioideae, are protandrous, releasing pollen from the fused anther tube throughout the initial male phase until the bilobed stigma emerges from the end of the tube, unfolds, and becomes receptive. Flowers last approximately one week, with each phase lasting several days.

Our focal species are all endemic to cloud forests of the Bolivian Andes. Two of the species, *C. brittonianus* and *C. mandonis*, occur at high elevations (e.g., 2700–3500 m); *C. brittonianus* is a narrow endemic whose entire range overlaps with *C. mandonis*, while *C. mandonis* has a wider distribution throughout Bolivia (Fig. 1). The third species, *C. incanus*, occurs at lower elevations (i.e., 1500–2800 m) that are parapatric to the other species (Fig. 1). Maps of each species' distribution were made with the R packages "maps" and "mapdata" using geographic coordinates of known localities of these species from taxonomically verified specimens in the Tropicos database (http://www.tropicos.org/). We performed fieldwork targeting the pollination biology of these species in December 2016 at two locations in the cloud forests of the department of La Paz, Bolivia: Valle del Zongo (~2200 m asl; 16°06′57.6″S, 68°04′48.0″W) for *C. incanus* and Chuspipata (~2700 m asl; 16°18′07.2″S, 67°48′54.0″W) for *C. brittonianus* and *C. mandonis*.

Quantifying pollinator importance

Observations were made at one to three flowers on each of five to eight individuals of each species to determine effective pollinators. Visits were recorded by placing video cameras with nightvision capabilities (HDR-CX550V and HDR-SR11; Sony, Tokyo, Japan) at specific flowers for 3–5 h periods during the day and night, resulting in 6–10 h of video per flower. Hummingbirds were targeted beginning at dawn (i.e., at 5 A.M.) and at dusk (i.e., around 5 P.M.), while bats were targeted after nightfall. Videos were reviewed at 3× speed using iMovie (Apple, Cupertino, California, USA) or with MotionMeerkat (Weinstein, 2015), an open source program that
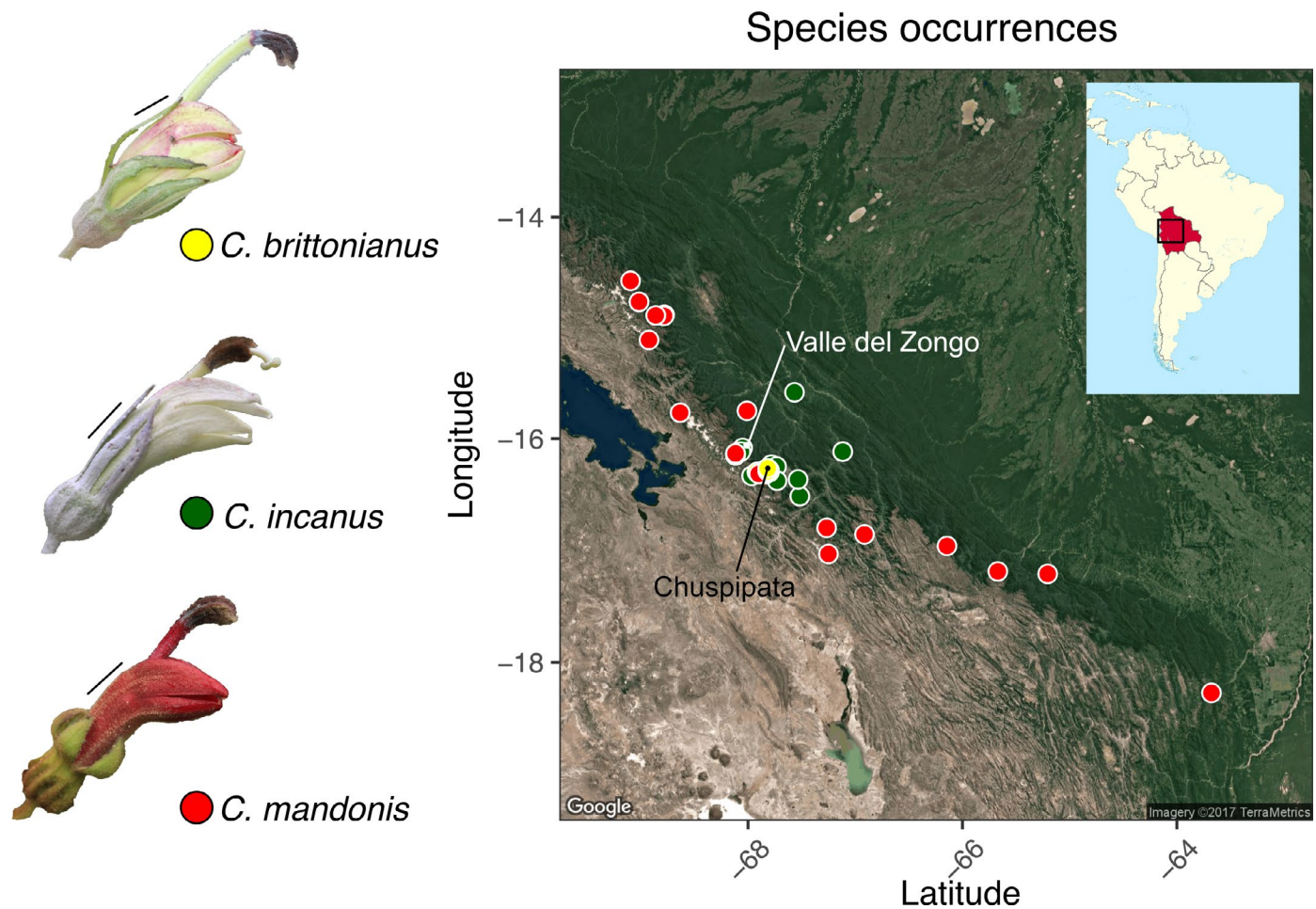
Species occurrences

FIGURE 1. Flowers and geographic distribution of the three focal *Centropogon* species, with points color coded according to species (green: *C. incanus*; yellow: *C. brittonianus*; red: *C. mandonis*). Sites where fieldwork was conducted, Chuspipata and Valle del Zongo, are indicated. A red rectangle in the inset map of South America shows the approximate location of the region depicted in satellite imagery. Scale bars next to flower images represent 1 cm.

uses computer vision technology to identify motion events from videos. For each video, we recorded the number, duration, and potential efficacy (i.e., whether or not the animal came into contact with the anther/stigma) of each visit, and used the results to calculate the visitation rate (i.e., number of visits/h) and the percentage of effective visits. Pollinator importance was then quantified as the product of pollination quantity (i.e., visitation rate) and quality (i.e., anther/stigma touch rate), scaled to 1.0 against the most effective pollinator within each species.

Pollen deposition

As an independent assessment of pollinator quality, we also quantified nocturnal and diurnal pollen receipt by flowers. We placed small squares (approximately 6 × 6 mm) of double-sided tape (476XL double-sided extended linear tape; 3M, Two Harbors, Minnesota, USA) on the reproductive organs of flowers at dusk and at dawn, and collected these on a microscope slide after 12 h (affixing samples to slides with a layer of single-sided tape; see Muchhala, 2006). Pollen receipt was quantified via light microscopy for each slide by counting a subsample of *Centropogon* pollen grains along a 6-mm-long transect through the center of the piece of double-sided

tape. Pollen grains could be identified to genus, but there was no visible difference between the pollen of the focal *Centropogon* species. While self-pollen deposition is possible, it is unlikely given the protandrous nature of the flowers of Lobelioideae in which the stigma does not begin to extend beyond the stamen tube and become receptive until pollen grains have been shed from the anthers. Mann-Whitney *U*-tests were used to determine whether there was a difference in the amount of pollen deposited diurnally and nocturnally for each of the three *Centropogon* species.

Quantification of floral traits

Seventeen quantitative floral traits were measured from one or two living flowers of 7–15 individuals per species, including corolla tube length and width, corolla opening width, and length of stamen exsertion (a full list of traits is shown in Table 1). Most of these traits were measured in Lagomarsino et al. (2017) and capture aspects of floral morphology known to be important to the pollination biology of centropogonid species. We used a linear discriminant analysis to summarize these measurements. Mean trait values were calculated for each trait, and a Tukey-Kramer post hoc test was performed to determine whether species have statistically significant different

TABLE 1. Average trait values of various floral measurements for three focal *Centropogon* species taken from flowers from living plants, with results from a Tukey-Kramer test demonstrating where the species are significantly different.

Species	C1	C2	W1	W2	SL	SW	PL	PW	TU	TW	ex2	AN	AW	AL	WB	WM	WT
C. brittonianus	40.7	25	6.6	9.4	29.6	3.5	113	2.5	24.3	14.4	42.7	66.6	4.1	13.5	11.8	15	15.8
C. incanus	50.8	28.8	26.1	11.5	30.8	4.1	119	2.5	29.3	14.1	23.2	55.7	4.1	14.2	10.4	12.2	16.6
C. mandonis	44.3	27.2	9.7	7.1	18.5	7	119	2.5	26.2	9.5	33.4	58.8	3.6	12.5	8.8	10.3	12.2
ANOVA results	$F_{2,30} = 16.39$	$F_{2,30} = 3.06$	$F_{2,30} = 26.96$	$F_{2,30} = 11.82$	$F_{2,30} = 16.19$	$F_{2,30} = 28.80$	$F_{2,30} = 0.14$	$F_{2,30} = 0.013$	$F_{2,30} = 4.88$	$F_{2,30} = 16.81$	$F_{2,30} = 33.02$	$F_{2,30} = 12.80$	$F_{2,30} = 3.99$	$F_{2,30} = 2.89$	$F_{2,30} = 28.11$	$F_{2,30} = 29.43$	$F_{2,30} = 6.12$
	$P < 0.001$	$P = 0.061$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.87$	$P = 0.97$	$P = 0.015$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.029$	$P = 0.071$	$P < 0.001$	$P < 0.001$	$P = 0.006$
Tukey-Kramer test results	b-i***, i-m***	N/A	b-i***, i-m***	m-b*, m-i***	m-b***, m-i***	m-b***, m-i***	N/A	N/A	i-b*	m-b***, m-i***	i-b***, m-b***, m-i***	i-b***, m-b***	N/A	N/A	i-b*, m-b***, m-i***	i-b***, m-b***, m-i*	m-b*, m-i*

Notes: All measurements in millimeters. C1: Greatest corolla length (base to end of lobes); C2: lesser corolla length (base to dorsal-ventral corolla lobe split); W1: greatest corolla width (base to dorsal-ventral corolla lobe split); W2: corolla width at dorsal-ventral corolla lobe split (i.e, functional width); SL: sepal length; SW: sepal width; PL: pedicel length; PW: pedicel width; TU: corolla tube width; TW: great corolla tube length; ex2: exsertion of stamen from corolla; AN: anther-nectar distance; AW: anther width; AL: anther length; WB: width of corolla base; WM: width of corolla at midpoint; WT: width of corolla at apex. In Tukey-Kramer test abbreviations, b = *C. brittonianus*; i = *C. incanus*; m = *C. mandonis*. *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$; N/A refers to traits for which a one-way ANOVA found no evidence that trait values differ between the species at $P < 0.01$.

mean values for each trait after confirming that a difference between means in the group was supported by a one-way analysis of variance (ANOVA).

We quantified floral color from living flowers with a Jaz spectrometer (Ocean Optics, Dunedin, Florida, USA) that measures wavelengths of light from the near UV through the near infrared (i.e., 250–800 nm). We took color measurements at the midpoint of the corolla tube for each individual for which morphology was quantified. Spectrometry data were analyzed in the "pavo" package of R (Maia et al., 2019).

Additionally, we measured stamen exsertion from 58 herbarium specimens (Appendix S1), spanning the known geographic range of all three species, at the Missouri Botanical Garden (herbarium code: MO) and the National Herbarium of Bolivia (LPB). This trait is potentially relevant to competition via interspecific pollen transfer, as it determines the site of pollen placement, and we thus wanted to explore whether it differs in regions of sympatry and allopatry in a pattern consistent with reproductive character displacement. All *C. brittonianus* were sympatric with *C. mandonis*, and all populations of *C. mandonis* in localities where *C. brittonianus* has never been collected were considered allopatric. Exsertion was measured as the straight-line distance between the split between the two dorsal corolla lobes to the apex of the dorsal anthers. Statistical signficance in exsertion length between the species was tested via *t*-test. We did not include measurements made from fresh tissue in this analysis.

RESULTS

Pollination observations

We documented a total of 73 visits over 151.5 h of video: 24 at *C. incanus*, 23 at *C. brittonianus*, and 26 at *C. mandonis*. These included both hummingbird and bat visits to each of the three species (Fig. 2). However, there are important differences in the frequency of these visits (Table 2). First, bat visits are much more common than hummingbird visits at *C. incanus* (0.75 vs. 0.18 visits/h), while the reverse is true in the other two species (*C. brittonianus*: 0.30 vs. 0.66 visits/h; *C. mandonis*: 0.39 vs. 0.81 visits/h). Second, the pollinator types differ in their efficacy in terms of the percentage of visits in which reproductive organs were contacted: across the three species, hummingbirds contacted anther tubes in 24–40% of their visits, whereas bats contacted anther tubes in 100% of their visits. As predicted based on aspects of its floral display that match the hummingbird pollination syndrome (e.g., red, narrow flowers), hummingbirds contacted anther tubes most frequently at the red-flowered *C. mandonis* (Table 2).

We also documented a difference in contact rate between species of hummingbird. Specifically, *C. brittonianus* and *C. mandonis* flowers in Chuspipata were visited by the relatively large violet-throated starfrontlet (*Coeligena violifer*), which contacted anther tubes 58.3% of the time, and the smaller amethyst-throated sunangel (*Heliangelus amethysticollis*), which contacted anther tubes only 8.3% of the time. The mechanism by which these hummingbirds failed to contact tubes, and thus acted as nectar robbers, differed between species: *C. violifer* often entered the relatively wide flower of *Centropogon mandonis* at extreme angles (video in Appendix S2a), while *H. amethysticollis* was too small, overall, to reach the anther tube in most cases (video in Appendix S2b). *Centropogon incanus* was visited only by a single species of
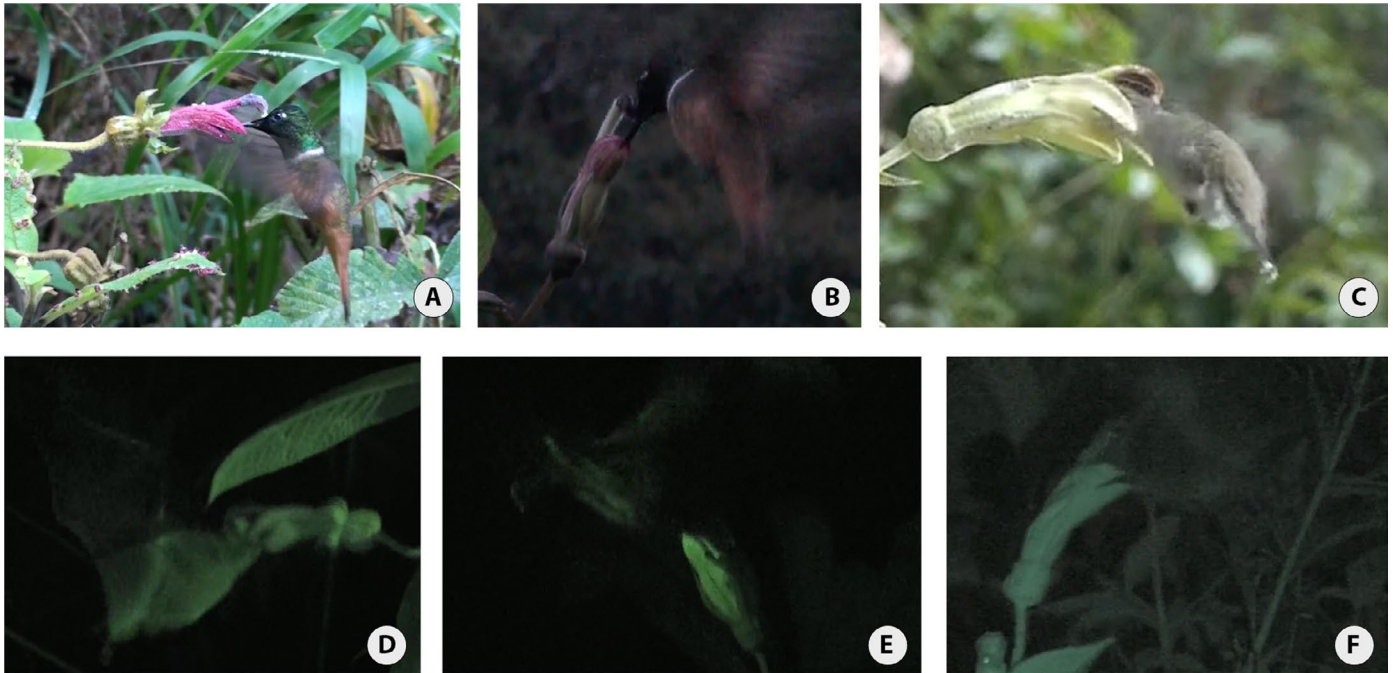
FIGURE 2. Snapshots from videos demonstrating effective pollination by each pollinator type at the three focal species. (A, B) *Heliangelus amethysticollis* visits *Centropogon mandonis* (A) and *C. brittonianus* (B). Note that in B, the hummingbird places its bill in a small hole created by appressed corolla lobes, which open after a bat visit. (C) *Thalurania furcata* visits *C. incanus*. (D–F) *Anoura* sp. visits *C. mandonis* (D), *C. brittonianus* (E), and *C. incanus* (F).

TABLE 2. Pollinator efficacy at flowers of *Centropogon brittonianus*, *C. incanus*, and *C. mandonis*: visitation rate of hummingbirds and bats (± SE), overall percentage of visits in which the flower's reproductive organs were touched, and the relative importance of each of these pollinators calculated as the scaled product of the average visitation and contact rates.

	Visitation rate (visits/h)		Contact rate		Relative importance	
	Hummingbird	**Bat**	**Hummingbird**	**Bat**	**Hummingbird**	**Bat**
C. incanus	0.18 ± 0.08	0.75 ± 0.25	28.6%	100%	0.064	0.936
C. brittonianus	0.66 ± 0.38	0.42 ± 0.12	23.8%	100%	0.275	0.725
C. mandonis	0.81 ± 0.31	0.39 ± 0.22	40.0%	100%	0.455	0.545

hummingbird (the fork-tailed woodnymph, *Thalurania furcata*), which contacted reproductive organs in 28.6% of visits.

Nectar-feeding bats cannot be identified reliably from videos, but the visits we documented were likely made primarily by *Anoura geoffroyi*. Bats of this genus can be distinguished from all other nectar bats in their lack of a uropatagium (the skin membrane connecting the legs), and in video clips where the back legs were visible none had a uropatagium (e.g., video in Appendix S2c). Only *Anoura* are known to occur in higher montane forests, and *A. geoffroyi* is by far the most common above 1500 m (Ortega and Alarcón-D, 2008), although *A. caudifer* or *A. cultrata* may also occur in our study sites.

Together, our results suggest a gradient of pollination specialization in these three species of peruvianid *Centropogon*, from nearly complete dependence on bat pollination to balanced generalization (i.e., approximately equal reliance on hummingbird and bat pollinators). Bats were the most important pollinator for both white-flowered *C. incanus* (93.6% of this species' pollinator importance; Table 2) and cream-flowered *C. brittonianus* (72.5% importance), though the latter species also had more than a quarter of estimated pollination services provided by hummingbirds (Table 2). Bats were a less important pollinator for the red-flowered *C. mandonis* (54.5%

importance), and this species can be considered a balanced generalist (Table 2).

Pollen deposition

Patterns of diurnal and nocturnal pollen deposition were largely congruent with our findings of relative importance based on visitation and contact rates. While we found that more pollen transfer occurred during the night (i.e., most likely by bats) than during the day (i.e., most likely by hummingbirds) at each of the three *Centropogon* species (Mann-Whitney *U*-test *P* values: 0.0012 [*C. mandonis*], 0.03515 [*C. incanus*], 0.0256 [*C. brittonianus*]), we see that diurnal pollen deposition still makes up more than a third of pollen receipt for *C. mandonis*, the balanced generalist (Table 3).

Quantification of floral traits

A linear discriminant analysis separates the three *Centropogon* species into distinct regions of morphospace (Fig. 3). The most important traits to separate taxa in LD1 (which explained 85.33% of between-species variance and primarily separated *C. incanus* from

TABLE 3. Average (± SE) number of pollen grains deposited along 6 mm transects of double-sided tape placed on stigmas during either a diurnal or nocturnal time period, with percentage of total pollen deposition and sample size in parentheses.

	Day	Night
Centropogon incanus	27.5 ± 7.7 (21.5%; n = 6)	100.3 ± 23.4 (78.5%; n = 8)
C. brittonianus	3.5 ± 1.2 (7.4%; n = 6)	43.5 ± 9.4 (92.6%; n = 6)
C. mandonis	73 ± 21.1 (36.3%; n = 3)	128 ± 37.1 (63.7%; n = 4)

the other two species) were midpoint width of the corolla tube, length of the corolla tube, anther length, and width of the corolla opening at the split between the dorsal and ventral corolla lobes (i.e., the functional corolla width); these all tended to be greater in *C. incanus*. The most important traits for LD2 (which explained the rest of the variance and primarily separated *C. mandonis* from *C. brittonianus*) were the width of the base of the corolla tube and the pedicel width. The average trait values for each species are provided in Table 1. The results from Tukey-Kramer tests show that two traits (stamen exsertion and the width of the corolla at its base) differ across all species, three traits (the entire length of the corolla and the greatest width of the corolla aperature) are significantly different in *C. incanus* in relation to the other two species, two traits (the distance of the anthers to the nectar chamber and the width of the corolla tube at its midpoint) are significantly different in *C. brittonianus* in relation to the other two species, and five traits distinguish *C. mandonis* from the other two species (the functional width of the flower, the length and width of sepals, the greatest corolla tube width, and the width of the corolla at its apex).

We generated average spectral curves for the corolla tubes of each species (Fig. 4). We found that *C. incanus*, a white-flowered species, reflects light across the visible spectrum, while the red-flowered *C. mandonis* primarily reflects above 600 nm (i.e., orange-red) within the visible spectrum. *Centropogon brittonianus*, which has cream-colored flowers that are mottled with red, are intermediate between these two species, as would be expected on the basis of human visual perception alone. Despite the fact that both hummingbirds and bats can see in the UV part of the spectrum (Winter et al., 2003), only the predominantly bat-pollinated *C. incanus* had any UV reflectance (i.e., <400 nm).

We measured exsertion length for 26 *C. brittonianus* and 32 *C. mandonis* herbarium specimens in order to study patterns of exsertion length in sympatry (with both species) or allopatry (with only *C. mandonis*—note that *C. brittonianus* never occurs alone; Fig. 1). Pooling all regions, stamen exsertion (mean ± SE) was significantly larger for *C. brittonianus* (44.9 ± 0.60 mm) than for *C. mandonis* (29.9 ± 0.56 mm; t_{57} = 8.35, P < 0.0001). Consistent with reproductive character displacement, we found that *C. mandonis* had significantly shorter exsertion in regions of sympatry with *C. brittonianus* than in regions of allopatry (28.2 ± 0.69 mm vs. 31.2 ± 0.68 mm; t_{30} = 3.02, P = 0.0005). The spread of the stamen exsertion lengths is shown in Figure 5.

DISCUSSION

We used a series of field-based techniques to examine the pollination biology of three closely related *Centropogon* species in close proximity in montane cloud forests of the La Paz Department of Bolivia. We found a gradient from primarily bat pollinated to balanced generalization on both bats and hummingbirds: the white-green flowers of *C. incanus* are primarily bat pollinated, the cream-colored to maroonish flowers of *C. brittonianus* are primarily bat and secondarily hummingbird pollinated, and the red flowers of *C. mandonis* are pollinated by bats and hummingbirds in roughly equal proportions. These three species may limit competition for pollination by differential reliance on bat and hummingbird pollinators. Additionally, the
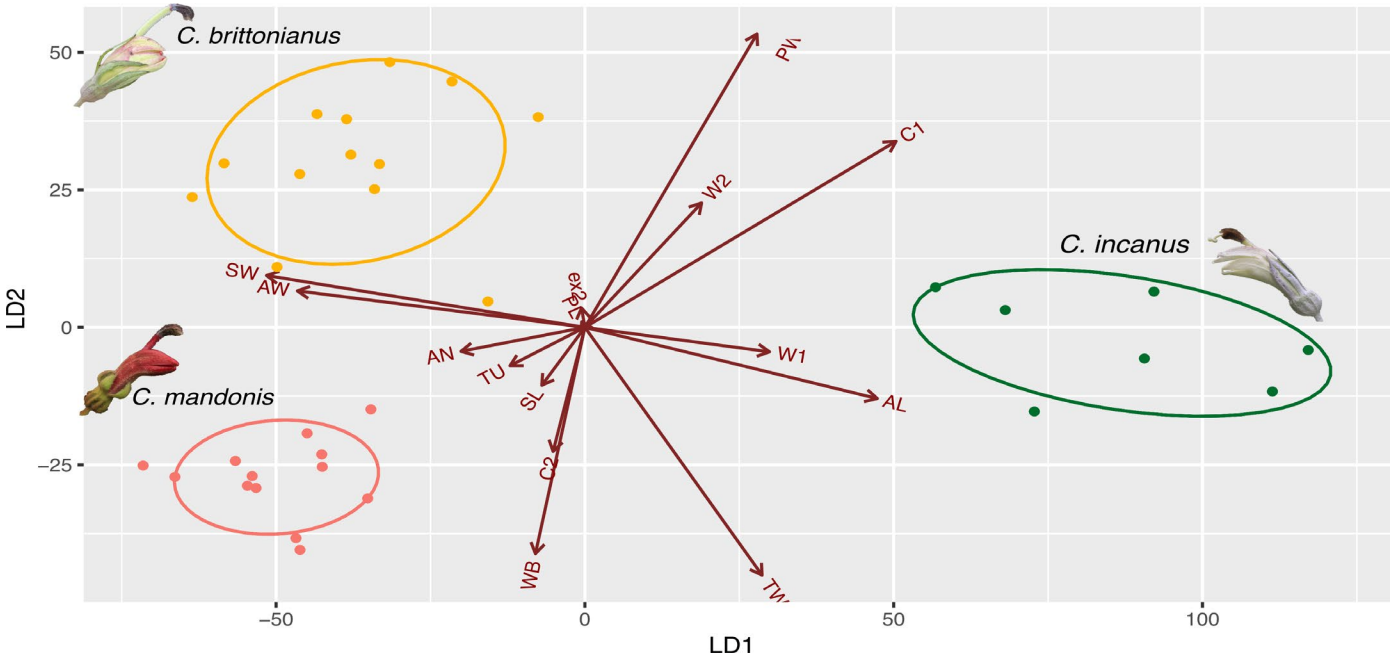

FIGURE 3. Linear discriminant analysis of morphological data, with data points color coded according to *Centropogon* species (green: *C. incanus*; yellow: *C. brittonianus*; red: *C. mandonis*). Traits noted by the biplots are defined in Table 1.
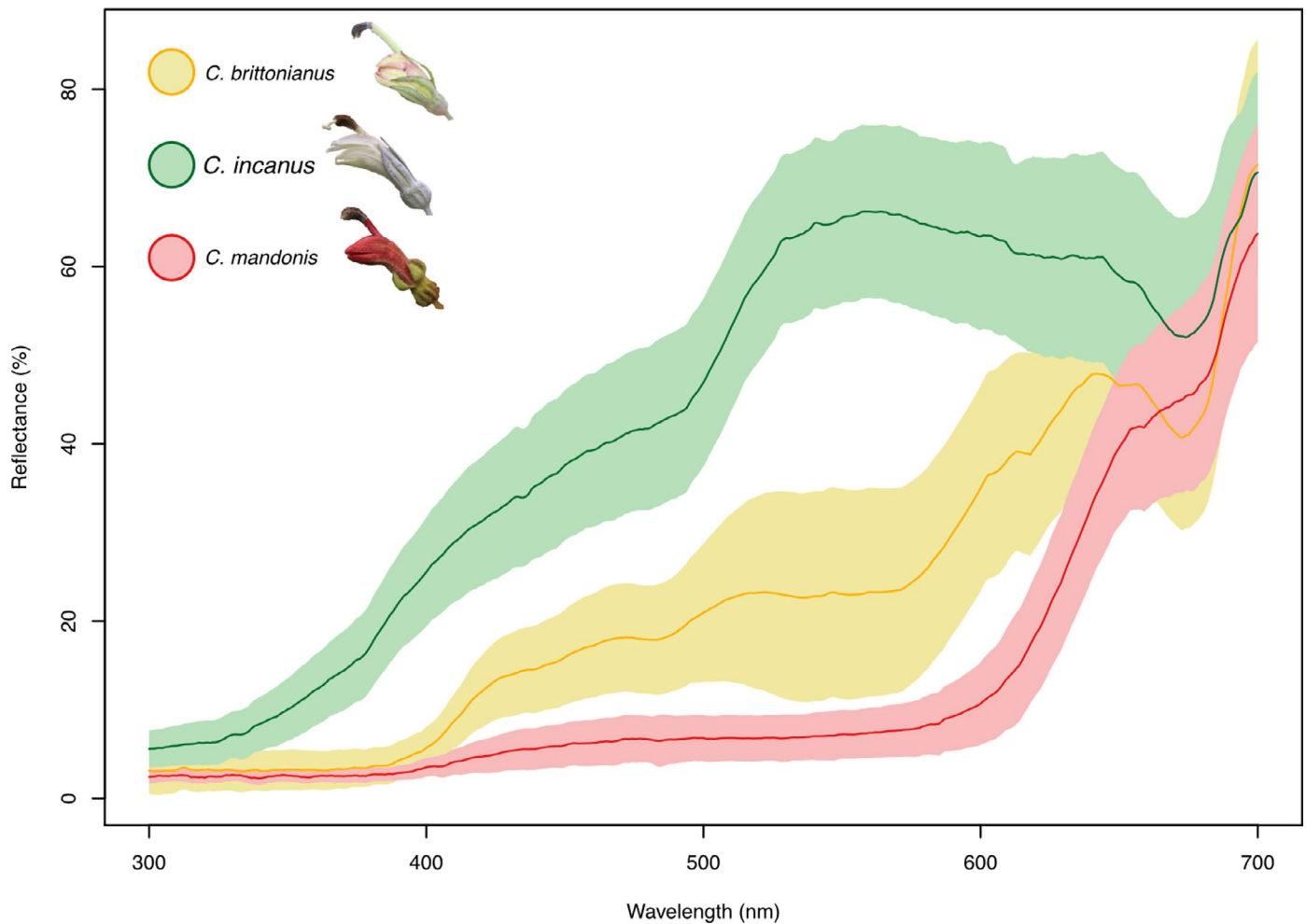
FIGURE 4. Spectral data from each of three *Centropogon* species, with average line and standard error shaded according to species (green: *C. incanus*; yellow: *C. brittonianus*; red: *C. mandonis*).

sympatric species *C. mandonis* and *C. brittonianus* have significantly different stamen exsertion lengths, further reducing competition by minimizing interspecific pollen transfer in regions of sympatry.

There were key differences in pollination efficacy between the two pollinator types. We found that the per visit efficacy of bats is much higher than that of hummingbirds for all three species, consistent with previous studies (Muchhala and Thomson, 2010). When bats visited, they always made contact with the reproductive organs of the flower, whereas hummingbirds were nectar robbers most of the time, obtaining nectar without contacting stamens/stigmas in 60–76.2% of their visits (with the highest contact rate for the red-flowered *C. mandonis*). This difference can also be seen in pollen deposition, which was always higher at night than during the day (Table 3). The combination of differences in visit rate and pollen transfer per visit results in the gradient of pollination specialization across the species (Table 2).

Specialization and pollination syndromes

Given the floral morphology of the three species, including intermediate floral traits in *C. brittonianus*, our pollinator observations support a role of pollinators as agents of selection in proportion to their importance to the reproduction of the plant. As in previous studies of the centropogonid clade (Muchhala, 2006; Lagomarsino et al., 2017), floral traits of our focal species suggest that these species are either chiropterophilous or ornithophilous. This was supported by our pollination observations: we documented that hummingbirds are the only diurnal visitors and bats are the only nocturnal visitors. As predicted on the basis of a recent macroevolutionary study of pollination syndromes across the centropogonid clade (Lagomarsino et al., 2017), we find that both of the species with pale flowers and wide floral apertures are primarily visited by bats, while hummingbirds are a relatively more important pollinator type for the red-flowered species with a narrower floral aperture. However, we find that no species is exclusively pollinated by either bats or hummingbirds (Table 2), highlighting potential oversimplification when pollination syndromes are assumed to be discrete (Waser et al., 1996; Smith et al., 2009; Ollerton et al., 2015).

Our results are largely consistent with the predictive nature of pollination syndromes (Fenster et al., 2004; Martén-Rodríguez et al., 2009; Rosas-Guerrero et al., 2014). For example, the generalist nature of *C. mandonis* was predicted in a study of pollination syndrome evolution across the entire centropogonid clade (Lagomarsino et al., 2017). This species was inferred to have a mixed pollination syndrome: while its floral morphology fell into
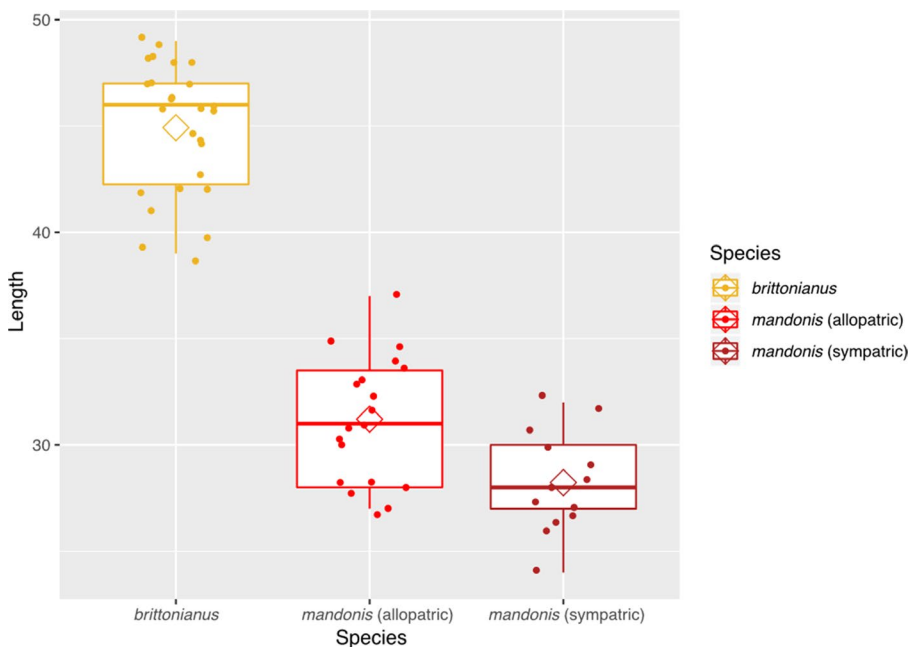
FIGURE 5. Box plots of stamen exsertion lengths in *Centropogon brittonianus* and *C. mandonis*, both in sympatry and allopatry with *C. brittonianus*. Individual measurements represented by dots, with mean values depicted by the diamond symbol. Measurements taken from herbarium specimens, in millimeters.

a "bat region" of morphospace, its bright red flower color predicted hummingbird pollination. *Centropogon mandonis* clustered with bat-pollinated species in a phylogenetic principal component analysis, primarily due to traits associated with the second PC axis, including the absence of a basal corolla constriction, a relatively wide floral opening, and large anthers—traits that are shared with *C. brittonianus* and *C. incanus*. Using measurements of living flowers of our focal species, we found that multiple measures of the floral width of *C. mandonis*—internal corolla width (W2 in Table 1), the width of the corolla at its apex (WT), and the greatest corolla tube width (TW)—are significantly narrower for *C. mandonis* than for the primarily bat-specialized *C. brittonianus* and *C. incanus*, consistent with predictions based on pollination syndromes (i.e., that hummingbirds are more effective at narrow flowers than bats; Muchhala, 2007; Temeles et al., 2002). However, flowers of *C. mandonis* are still much wider than a typical hummingbird-pollinated flower, which likely allows effective pollination by both functional groups of pollinators. Flowers with intermediate morphology and mixed pollination syndromes that allow them to be effectively pollinated by both hummingbirds and bats have been documented elsewhere in the centropogonid clade in *Siphocampylus sulfureus* (Sazima et al., 1994), as well as in Antillean Gesneriaceae (Martén-Rodríguez et al., 2009), *Aphelandra acanthus* (Muchhala et al., 2009), and Brazilian *Abutilon* species (Buzato et al., 1994). Generalized pollination is sometimes associated with specific shape traits that allow effective usage by multiple classes of specialized pollinator, such as a corolla constriction above the nectar chamber in Antillean Gesneriaceae that directs hummingbird bills to contact stamens and stigmas in flowers that are primarily bat pollinated and would otherwise be too wide for effective pollination by hummingbirds (Martén-Rodríguez et al., 2009). However, the gross morphology of all three species of Bolivian *Centropogon* is similar, and size differences in key traits such as the width of the corolla and the

exsertion of the anthers seem to drive the differences in pollinator efficacy across the three species.

As in *C. mandonis*, hummingbirds are important pollinators of *C. brittonianus*, though bats are this species' primary pollinator. Floral traits of *C. brittonianus* place it comfortably within the bat pollination syndrome, but this species relies on an idiosyncratic aspect of floral anthesis in order to also exploit hummingbird visits effectively. When the buds initially open, the margins of the corolla lobes remain pressed close together, with only the distal tips separating. As predicted from field and empirical studies (Temeles et al., 2002; Muchhala, 2003, 2007), this narrow opening allows effective pollination by the hummingbird *Coeligena violifer* (although not by the smaller-bodied *Heliangelus amethysticollis*). The first bat visit to a flower fully separates the corolla lobes, precluding further effective hummingbird pollination since the wider floral aperture precludes contact with reproductive organs. As a result, hummingbirds that visit after corolla lobe separation have a high chance of operating as nectar robbers. This morphology allows flowers to still closely fit bat heads but also provides a fail-safe in the event that bats do not visit a particular flower.

Flower color is an important component of pollination syndromes (at least as they present in the centropogonid clade) and may be important in attracting pollinators in this Bolivian system. The bright red floral color of *Centropogon mandonis* seems to have arisen recently from dull-colored relatives (Lagomarsino et al., 2017), which include the sympatric, primarily bat-pollinated *C. brittonianus*. This red is highly visible to diurnal hummingbirds, which produce four types of cone photoreceptors and have very acute visual perception (Bennett and Cuthill, 1994), and less so to nocturnal bats, which have two cone types and dichromatic vision (Winter et al., 2003; Müller et al., 2009). On the other hand, cream-colored flowers of *C. brittonianus* provide contrast at night that may make these flowers more visible to bats, their primary pollinators. Other species in the community at Chuspipata (where *C. brittonianus* and *C. mandonis* co-occur) share these color cues, including brightly colored flowers of species with traits associated with the hummingbird pollination syndrome in groups as diverse as *Barnedesia* (Asteraceae), *Cavendishia* (Ericaceae), *Fuchsia* (Onagraceae), and multiple species of Gesneriaceae, as well as species that display traits associated with bat pollination including dully colored flowers, such as *Macrocarpea* (Gentianaceae), *Marcgraviastrum* (Marcgraviaceae), *Cobaea* (Polemoniaceae), and *Condaminea* (Rubiaceae).

Character displacement and shared pollinators in sympatry/parapatry

Because these three closely related *Centropogon* species occur in close proximity to each other and share pollinators, mechanisms that reduce interspecific pollen transfer are important. This is particularly true considering that two species are either sympatric for either the entirety of their range (*C. brittonianus*, which is limited to a small area in the La Paz Department) or part of it (*C. mandonis*,

which can be found throughout mid-elevations in Bolivia), while the third species, *C. incanus*, is parapatric to the other species throughout its range, where it is found ca. 400–700 m lower on the same slopes. These short distances between species likely do not preclude pollination by the same individual pollinators, even if it may be relatively rare between *C. incanus* and the other two species. That these species maintain distinct morphologies in close proximity suggests that either current barriers to gene flow are strong, or that there is strong selection against hybrids.

We provide evidence for reproductive character displacement that likely reduces interspecific pollen transfer between the sympatric and co-flowering *C. mandonis* and *C. brittonianus*. Across their ranges, a 15 mm difference in exsertion length of reproductive organs results in pollen placement on different parts of their pollinators' bodies: our videos show that the shorter anther column of *C. mandonis* (29.9 mm) places pollen on the tops of bats' heads, whereas the longer column of *C. brittonianus* (44.9 mm) places pollen further back, between bats' shoulder blades. This allows these two closely related species to reduce costs associated with sharing pollinators. This difference in pollen placement is accentuated in zones of sympatry: where *C. mandonis* co-occurs with *C. brittonianus*, its exsertion length is 3 mm shorter than throughout the rest of its range, a reduction of ~10%. Because *C. mandonis* and *C. brittonianus* share bats as pollinators, this difference likely serves to limit the negative effects of interspecific pollen transfer and/or stigma clogging in regions of sympatry. A similar pattern of character displacement has been shown among co-occurring *Burmeistera* species, which are also pollinated by nectar bats in the genus *Anoura* (Muchhala and Potts, 2007). Further, small differences in exsertion length (i.e., <6 mm) can have major functional consequences in the pollination of *Burmeistera*, a group closely related to *Centropogon* with extremely similar pollination and floral biology (Muchhala and Potts, 2007; Muchhala, 2008). While the stamen exsertion differences we identify in *C. mandonis* and *C. brittonianus* are consistent with character displacement in the same pattern that has been experimentally confirmed in close relatives, it is possible that these differences instead reflect unmeasured parameters, including differences in the abiotic environment and stochasticity. Future studies could test experimentally for character displacement in this Bolivian system, as well as examine the consequences of differences in stamen exsertion for maintaining species boundaries between these recently diverged taxa.

CONCLUSIONS

By targeting a group of understudied tropical plants, we were able to gain insights into concepts in plant reproductive biology: pollination syndromes and character displacement. We documented a gradient from specialist to balanced generalist pollination. The relative difference in the importance of pollinators in this system is tied to floral traits, with species with more chiropterophilous traits relying more on bats as pollinators and species with more ornithophilous traits relying more on birds. This points to one of the failings of the pollination syndrome concept, as currently conceived: that it is frequently interpreted to be strictly categorical in nature. However, this study and others document that plants can have a mix of traits from different pollination syndromes, reflecting diverse selection agents shaping floral morphology and resulting in flowers that effectively utilize pollinators of multiple classes. Our results also suggest that competition for pollination among these three closely

related *Centropogon* species that occur in geographic proximity is reduced by differences in traits that determine pollen placement on a pollinator's body, a pattern that was exaggerated in regions in sympatry. This represents one of the few potential cases of character displacement in plants, though future studies are necessary to confirm that all criteria defining character displacement are met (Beans, 2014). Together, these results have the potential to shed light into the hyper-diversity of plant taxa in Andean cloud forests. Future work that characterizes gene flow and population structure of these species will clarify the importance of current and past introgression in the evolutionary history of these three taxa and provide fundamental insights to the process of speciation in the face of shared pollinators and overlapping distributions in this species-rich clade.

SUPPORTING INFORMATION

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

APPENDIX S1. Voucher information for herbarium specimen measurements.

APPENDIX S2. Video examples of pollination events.

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