Premating barriers prevent gene flow between co-occurring bat-pollinated bromeliads in a montane forest

Stephanie Núñez-Hidalgo[1] & Alfredo Cascante-Marín[2]

[1] Sistema de Estudios de Posgrado, Universidad de Costa Rica, San Pedro de Montes de Oca. Present address: Escuela de Ciencias Ambientales, Universidad Nacional, Heredia, Costa Rica.

[2] Centro de Investigaciones en Biodiversidad y Ecología Tropical (CIBET) & Escuela de Biología, Universidad de Costa Rica, 11501-2060 San Pedro de Montes de Oca, San José, Costa Rica. https://orcid.org/0000-0001-6382-9316.

Corresponding Author:

Alfredo Cascante-Marín

San Pedro de Montes de Oca, San José, 11501-2060, Costa Rica

Email address: alfredo.cascante@ucr.ac.cr

Abstract

Background. Reproductive isolation mechanisms in flowering plants are fundamental to preserving species' evolutionary independence and to enabling the local coexistence of closely related species. These reproductive barriers are expected to contribute to maintaining local diversity of highly diverse plant guilds, such as bromeliads in neotropical ecosystems. We evaluated how strong and effective these barriers are by analyzing different mechanisms that act before and after pollination in a guild of four epiphytic bromeliads from the genus *Werauhia* (Tillandsioideae) pollinated by bats in a Costa Rican montane forest.

Methods. We employed several reproductive isolation indices proposed in the literature to estimate the effect of flowering phenology, floral morphology, interspecific compatibility, production, and viability of hybrid seeds as barriers to gene flow between species pairs.

Results. The overall reproductive isolation between species was complete or nearly so. We found that temporal isolation due to different flowering schedules between species significantly contributed to preventing interspecific gene flow. However, flowering data from four reproductive seasons showed interannual variation in the intensity of this temporal barrier due to fluctuations in the species' blooming patterns. For species with overlapping flowering, mechanical isolation caused by differences in flower size and anther position was significant, and such differences in flower architecture are thought to influence pollen deposition on different areas of the pollinator´s body. Postmating barriers showed varying intensity, from full to partial interspecific incompatibility. When hybrid progeny was produced, the number of seeds and their germination capacity were lower compared to progeny from conspecific crosses.

Conclusions. Overall, pre-pollination mechanisms (phenology and floral design) were of great importance to eliminate pollen transfer between species and, when present, postmating barriers had a redundant effect. Our results contradict previous reports that suggested a weak effect of premating barriers among bromeliad species. Additional studies involving other pollination guilds are required to gain a better understanding of the prevalence of different reproductive isolation mechanisms in the highly diverse Bromeliaceae family.

Introduction

Reproductive isolation is a fundamental driver of plant biodiversity (Baack et al., 2015) by preventing reproductive interference and facilitating the simultaneous coexistence of closely related species (Schemske, 2010). Closely related species that share the same habitat frequently employ a variety of reproductive isolation mechanisms to prevent interspecific pollen transfer or hybridization (Coyne & Orr, 2004; Lowry et al., 2008; Widmer et al., 2009), which may result in the loss of gametes and the formation of nonviable hybrids (Campbell & Aldridge, 2006; Moreira-Hernández & Muchhala, 2019).

Reproductive isolation mechanisms restrict gene flow between species and consist of floral differences of a morphological, etological, physiological, or genetic nature and can be classified into two types according to whether they occur before (premating) or after (postmating) pollination (Campbell & Aldridge, 2006; Levin, 1971). The degree of premating and postmating isolation can vary among species and may be influenced by the pollination system (Cozzolino et al., 2004; Cozzolino & Scopece, 2008). Generally, the efficiency of reproductive isolation mechanisms is complemented sequentially at each stage, that is, a reproductive barrier prevents gene flow that was not eliminated by previous barriers (Widmer et al., 2009).

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Based on evidence from the past 20 years (Christie et al., 2022; Lowry et al., 2008), premating barriers appear to be significantly more effective than postmating barriers, with floral isolation mechanisms being more robust. Most published data on reproductive isolation barriers (82%) are from temperate plant groups that mainly include herbaceous and perennial species from the Orobanchaceae and Orchidaceae families pollinated by insects (Christie et al., 2022; Schiestl & Schlüter, 2009). Reproductive barriers operating in neotropical plant lineages with specialized pollination systems are poorly understood and have only been the subject of recent investigations (e.g., Albuquerque-Lima et al., 2024; Arida et al., 2021; Cuevas et al., 2018; Kay, 2006; Ramírez-Aguirre et al., 2019). Understanding the species coexistence and maintenance of plant diversity in highly diverse tropical ecosystems requires further comparative studies of reproductive isolation mechanisms.

The Bromeliaceae family is an example of a highly diverse lineage (ca. 3600 species) that is almost exclusive to the American Continent (except for one species from West Africa) (Benzing, 2000). Bromeliad diversity is concentrated in four areas: the Atlantic Forest in eastern Brazil, the Andean slopes, Central America, and the Guiana Highlands (Zizka et al., 2020). The great morphological diversity in bromeliads is partially ascribed to hybridization processes that have also contributed to speciation (Gardner, 1984; Goetze et al., 2017; Schulte et al., 2010). However, even in the presence of potential hybridization, the maintenance of high regional and local diversity in bromeliads implies the existence of reproductive isolation mechanisms.

To understand how reproductive coexistence operates in co-occurring congeneric species of bromeliads and how local plant diversity is maintained, we estimated the strength and relative contribution of several pre- and postmating reproductive barriers in a group of four sympatric *Werauhia* species in a montane forest in Costa Rica. The mountains of southern Central America extending from Costa Rica to western Panama represent the radiation center of the genus *Werauhia* J. R. Grant from subfamily Tillandsioideae (Grant, 1995). This group of epiphytic and tank-forming bromeliads consists of approximately 100 species (Gouda & Butcher, cont. updated) and is distinguished by a combination of nocturnal anthesis, inconspicuous floral coloration (white, cream or greenish), petals with dactyloid-shaped basal appendages with divided apex, and cupular-shaped stigmas without papillae (Grant, 1995). *Werauhia* has been retrieved as monophyletic in molecular investigations (Barfuss et al., 2005) and appears to have a relatively recent diversification history (about 5 million years) (Givnish et al., 2011). Understanding the ecological factors that modulate the species´ reproductive coexistence may help elucidate the mechanisms driving their diversification.

Bromeliads are plants of ornamental interest that have been used to develop and cultivate artificial hybrids (Negrelle et al., 2012). This suggests that post-pollination mechanisms such as interspecific genetic incompatibility or incongruity (sensu Knox et al., 1986) might not represent an important reproductive barrier in the family. Nonetheless, the rarity of naturally occurring hybrids (Benzing, 2000; Gardner, 1984; Neri et al., 2018; Smith & Downs, 1974; Souza et al., 2017) instead suggests that premating barriers could be more effective. However, some authors have advocated the contrary view that bromeliads have inadequate prepollination barriers (Wendt et al., 2008).

This study evaluated four mechanisms of reproductive isolation in sympatry acting before and after pollination: (i) temporal barriers related to population floral phenology, (ii) mechanical floral barriers associated with flower size and position of reproductive organs, (iii) prezygotic barriers related to interspecific incompatibility or incongruity, and (iv) postzygotic barriers related to the production and viability of hybrid seeds. By using a series of indirect methods or Reproductive Isolation Indices (RI) described by Sobel and Chen (2014), we estimated how much gene flow is reduced by each reproductive barrier and quantified the relative contribution of pre- and postmating barriers to total reproductive isolation between species pairs.

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Materials & Methods

Study site

The bromeliads studied were located at a montane forest ecosystem in the Central Valley of Costa Rica (9º 52'– 9º 54" N and 83º 57'– 84º 00' W). La Carpintera Protective Zone is a small mountain formation between 1500 and 1850 m asl with nearly 2400 hectares in size (35% primary forest and 57% old secondary forest) (Sánchez-González et al., 2008). The zone receives

an average annual rainfall of 1839.2 mm, with a mean temperature of 16.1 °C, and a mild drier season runs from December to April (Rios & Cascante-Marín, 2017); however, the presence of fog is frequent during the night and early morning. The Life Zones System of Holdridge (1978) classifies the vegetation as both humid and very humid lower montane forest. The local diversity of vascular epiphytic plants represents nearly one-third of the local flora, and bromeliads contribute with 29 species; the genus *Werauhia* stands out for its abundance and diversity (eight species) (Sánchez-González et al., 2008).

Study species

We selected the four most abundant *Werauhia* species at the study site: *W. ampla* (L. B. Sm.) J. R. Grant, *W. nephrolepis* (L. B. Sm. & Pittendr.) J. R. Grant, *W. pedicellata* (Mez & Wercklé) J. R. Grant, and *W. subsecunda* (Wittm.) J. R. Grant. These epiphytic species develop small to medium-sized rosettes, simple spiked (*W. ampla* and *W. subsecunda*), or compound inflorescences (*W. nephrolepis* and *W. pedicellata*) (Fig. 1). These bromeliads share the same pollinator at the study site, the nectar-feeding bat *Hylonycteris underwoodi* (Phyllostomidae) and exhibit a highly self-compatible mating system with an autonomous delayed selfing mechanism (Núñez-Hidalgo & Cascante-Marín, 2024). Their geographic distribution mostly encompasses the very humid and cloudy forests between 1000 and 2750 m asl on the Talamanca Mountain range in Southern Central America between Costa Rica and Panama (Morales, 2003).

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Pre-mating mechanisms

Temporal isolation by floral phenology — We determined the flowering phenology pattern for each species along four non-consecutive reproductive seasons. For this, we established 10 sampling points with a high density of bromeliads at the study site (1650-1780 m asl) and conducted biweekly censuses to document the number of plants opening flowers from a sample of 70 to 385 plants per species. The censuses were carried out from October 2018 to July 2019 and January to July 2021. We incorporated data from the flowering seasons of 2012-2013 and 2014-2015 previously collected by the second author using the same methodology.

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Then, we estimated the RI arising from phenological differences (RI_F) between species pairs following the formula RI_{4S2} proposed by Sobel and Chen (2014) and included in the Excel spreadsheet template provided in their supplementary material. This index, hereafter called RI_F, reflects the magnitude of floral asynchrony as a barrier to the formation of hybrids between pairs of species and contemplates shared and unshared flowering days and differences in sample sizes. A RI_F value equal to zero indicates the absence of reproductive barriers, while a value equal to 1 corresponds to complete reproductive isolation. We estimated RI_F as a mean value from the four phenology periods.

Mechanical isolation by floral morphology — Plants may prevent or reduce interspecific pollen transfer by placing the pollen on different parts of the pollinator's body, and this is achieved through differences in flower size and position of reproductive organs in the corolla (Dressler, 1981; Muchhala, 2008). So, we develop two mechanical RI, one for differences in size (RI_{MS}) and other to variations in position (RI_{MA}) -Consequently, we measured the following floral morphology traits: (i) length of the corolla, (ii) diameter of the mouth of the corolla, (iii) length of the stamens and (iv) length of the pistil, using a ruler with a precision of one millimeter. The sample consisted of 33 freshly opened flowers from 20 *W. subsecunda* plants, 31 flowers from 15 *W. nephrolepis*, 30 flowers from 15 *W. ampla*, and 30 flowers from 10 *W. pedicellata*.

We used a MANOVA to test the significance of the differences in floral traits among species and a post-hoc Tukey test for species-pairs comparisons after a significant result with the built-in package of the R software. To visualize the differences in flower morphology among species in a multidimensional space, we used Principal Component Analysis (PCA) with the *FactoMineR* package (Lê et al., 2008) in the R software platform (R Core Team, 2021). Upon examining the PCA biplot, we determined that species exhibiting some overlap or unclear separation along either of the PCA dimensions (i.e., possessing similar floral morphologies) have the possibility of gene exchange, indicating a weak morphological barrier, hereafter called RI_{MS}. Consequently, we conservatively assigned a value of $RI_{MS} = 0$. If clearly separated in the multidimensional space (i.e., with different floral morphologies), we assigned a $RI_{MS} = 1$, indicating complete isolation due to floral size.

In most *Werauhia* species, the anthers position together forming a hood over the dorsal side of the corolla mouth or, less frequently, the anthers may separate into two groups of three (triplets) and locate on both sides of the corolla mouth with the stigma on either side (Utley, 1983). For species pairs sharing the same arrangement of reproductive structures, we assumed no restriction to gene flow and assigned a reproductive isolation index (RI_{MA}) value equal to 0 (no isolation), otherwise we assigned a value equal to 1 (complete isolation), since pollen deposition is expected to occur on different parts of the pollinator's body.

Post-mating mechanisms

To estimate the strength of post-pollination barriers, we followed the formula proposed by Sobel & Chen (2014): $RI_{4C} = 1\text{-}2(H / H + C)$, where H = hetero-specific events (percentage of fruits, seed number or seed germination from interspecific manual crosses), and C = conspecific events (percentage of fruits, number of seeds or seed germination from intraspecific manual crosses). We calculated the indices corresponding to inter-specific incompatibility or RI_I (pre-zygotic barrier), hybrid seed production or RI_S (post-zygotic barrier) and seed viability or RI_V (post-zygotic barrier). The RI value indicates the amount of interspecific gene flow at each stage,

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Interspecific incompatibility
Hybrid progeny and viability

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where -1 indicates the presence of inter-specific pollen flow (absence of barriers), 0 indicates random pollen flow, and 1 indicates complete isolation of gene flow between species. Data on conspecific events (manual cross-pollinations) were obtained from a related work on the breeding systems of the study species at the same site (Núñez-Hidalgo & Cascante-Marín, 2024).

Interspecific incompatibility — We conducted controlled interspecific cross-pollinations in a total of 67 plants (18 *W. ampla*, 19 *W. pedicellata,* and 29 *W. subsecunda*) from November 2018 to May 2019. These species showed an overlap in reproductive phenology during the study period and could potentially interbreed. The manipulated plants were kept in a shade house located at the study site in the premises of the Iztarú Field School of the Association of Guides and Scouts of Costa Rica at 1760 m asl. Interspecific manual pollinations were performed reciprocally; thus, a plant was both pollen-donor and pollen-recipient. Before floral anthesis, anthers were carefully removed with a pair of tweezers before dehiscence to avoid contamination of the stigma and stored in paper envelopes until the time of manual pollination in the same night. Pollen in sufficient quantity was applied to receptive stigmas (i.e., with stigmatic fluid present) 1–2 hours after anthesis using a metal spatula.

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Hybrid progeny and viability — To determine the existence of post-zygotic barriers acting on hybrid progeny formation, we counted the number of seeds per fruit from successful interspecific crosses and compared it to the respective intraspecific crosses. We tested the viability of the hybrid seeds by carrying out a germination test in laboratory conditions. We mixed the seeds from each species-pair cross and distributed a sample of 480 seeds among 12 replicates, each containing 40 seeds. Each replicate was germinated on wet paper towel in Petri dishes. To reduce the incidence of fungal contamination, we once applied a commercial fungicide (Vitabax 40 WP). We determined the number of germinated seeds twice a week, a seed had germinated when the radicle emerged from the seed coat. Once we noticed no seeds germinating, we calculated the cumulative percentage of germination, usually one month after the trial began.

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Contribution of each barrier type and total reproductive isolation

Each reproductive barrier contributes to isolation in proportion to the order in which they occur in the plant's life. As a result, the first barrier to action will reduce gene flow, implying a greater contribution to reproductive isolation (Coyne & Orr, 1989; Ramsey et al., 2003). We used the formulas proposed by Sobel & Chen (2014) and provided in their supplementary material (evo12362-sup-0003) to estimate the relative and absolute contribution of each type of barrier and total reproductive isolation between each pair of species. We also identified which category of isolation barrier (pre- or postpollination) contributes more to reducing gene flow

between species. The calculations were performed using an Excel spreadsheet provided by Sobel & Chen (2014) and included here as a "Supporting information" file.

Results

Isolation by floral phenology

The species exhibited an annual pattern of flowering (sensu Newstrom et al., 1994) but the intensity and distribution of flowering peaks varied among species and between years (Fig. 2). The blooming periods were seasonally divided; *W. ampla, W. pedicellata,* and *W. subsecunda* mostly bloomed in the dry season and *W. nephrolepis* flowered separately during the rainy season (Fig. 2). *Werauhia ampla* and *W. subsecunda* showed the longest reproductive periods (5–6 months), both with a pattern of constant intensity or "steady-state" (sensu Janzen, 1967) and flowering peaks of relatively low intensity (usually <40% of the observations) (Fig. 2). *W. pedicellata* showed a defined bimodal pattern and a low to moderate overlap with the two previous species. The flowering of *W. nephrolepis* was of short duration with a very marked peak resembling the "cornucopia type" pattern (sensu Janzen, 1967) and temporally isolated from the rest (Fig. 2).

Estimations of temporal isolation between species-pair combinations using the four-year average value of the index were very variable, ranging from 0.128 to 0.991 (Table 1). It was the lowest between *W. ampla* and *W. subsecunda* (RI_F = 0.128 and 0.258), and for all paired comparisons involving *W. nephrolepis*, it indicated strong temporal isolation in both directions ($RI_F \geq 0.97$) (Table 1). There were some variations between years in the strength of this barrier for some species pairs (Table S1).

Isolation by floral morphology

Differences in floral morphometry among species were statistically significant (MANOVA test: df = 3, 351; F-value = 28.358; p-value < 0.001). Similarly, all paired comparisons for each trait were significant (Tukey test; $p<0.01$). The length of pistil and stamens in *W. ampla* and *W. nephrolepis* were 2–3 times longer compared to *W. subsecunda* and *W. pedicellata* (Fig. 3A). The PCA suggested two groups based on dimensions of floral parts, large (*W. ampla* and *W. nephrolepis*) versus small-flowered (*W. subsecunda and W. pedicellata*) species (Fig. 3B). The first component explained most (92.48%) of the variation in the data, with stamens and pistil length showing the highest scores (Table S2). These size differences in reproductive structures between those two species groups were deemed to represent complete isolation (RI_M = 1; Table 1), since such morphological dissimilarity precludes effective interspecific pollen transfer.

The species *W. ampla, W. nephrolepis,* and *W. pedicellata* share the same position of stamens and stigma on the dorsal part of the corolla mouth (Fig. 4), suggesting the probability of gene flow (RI_{MA} = 0). In *W. subsecunda* the pistil and stamens separate into two triplets that locate in lateral position at the corolla mouth (Fig. 4C). This conformation of the reproductive organs represents a strong barrier to gene flow with respect to the other species, thus paired comparisons involving *W. subsecunda* were assigned a complete reproductive isolation for this aspect of floral morphology (RI_{MA} = 1) (Table 1).

Isolation by interspecific incompatibility

Reciprocal crosses between *W. subsecunda* and *W. pedicellata* showed partial interspecific incompatibility and resulted in fruit percentages of 37% and 47.8% (RI_I = 0.097 and 0.348, respectively; Table 2). Crosses involving *W. ampla* only produced fruits with *W. subsecunda,* when the latter species acted as pollen recipient (54.5%, RI_I = 0.168), and it represented a case of asymmetric incongruity. Because of full incompatibility, the reproductive barriers between *W. ampla* and *W. pedicellata* were complete (RI_I = 1).

Isolation by hybrid progeny unviability

When compared to the respective intraspecific crossings (Table 2), the number of hybrid seeds per fruit from reciprocal crosses between *W. subsecunda* and *W. pedicellata* resulted in a seeds reduction of 48% and 66.4%, respectively. This represented a relatively low to moderate isolation barrier between the two species (RIs = 0.316 and 0.414, respectively). When *W. subsecunda* (the pollen recipient) crossed with *W. ampla*, fruits produced about a third as many hybrid seeds as fruits from intraspecific crosses of the same species (214 vs. 636 seeds, respectively; Table 2). This represented a moderate barrier to reproduction (RI_V = 0.497).

The germination capacity of hybrid seeds from *W. subsecunda* sired with pollen from *W. pedicellata* was high (92.3%; Table 2). This resulted in a nearly absent isolation barrier (RIv = 0.03). On the contrary, hybrid seeds from *W. pedicellata* sired with pollen from *W. subsecunda* did not germinate as well (31.9% vs. 92.5% for intraspecific crosses) (Table 2) and represented a moderate isolation barrier (RIv = 0.487). Hybrid seeds from *W. subsecunda* and *W. ampla* (as pollen donor) had lower viability compared to seeds from intraspecific crosses of the latter species (73.1 vs. 98.8%; Table 2). This loss of viability represented a relatively weak isolation barrier (RIv = 0.149).

Discussion

Using standardized metrics or reproductive isolation indices (RI), this study presents novel

information on the strength and importance of several pre- and postmating barriers among syntopic species in the Bromeliaceae family. Previous studies on this plant group have only analyzed separate isolation barriers without employing comparative metrics. For instance, Wendt et al. (2008) studied premating barriers and suggested that they were ineffective at preventing interspecific gene flow, while Souza et al. (2017) found that postmating barriers related to interspecific incompatibility led to different levels of reproductive isolation. Our research shows that reproductive isolation between species was complete and that pre-pollination mechanisms were more relevant as reproductive barriers between chiropterophilous bromeliads of the genus *Werauhia* from subfamily Tillandsioideae.

The role of temporal barriers -- Research has shown that flowering time can have varying effects on plant reproductive isolation, ranging from minimal to significant (mean RI = 0.375, Christie et al., 2022). Our study found that differences in flowering time were an important barrier to gene flow among the *Werauhia* species studied, with a mean RI_F of 0.677. The overall strength of this barrier varied between years from 0.624 (2020-2021 season) to 0.712 (2012-2013 season), though indicating rather modest variation. Between species pairs, however, variation in isolation strength of flowering time ranged from as low as 0.128 to nearly complete isolation at 0.991, reflecting the diversity of flowering patterns at the study site. Similarly, the strength of this barrier varied among years between some species pairs (Table S1). This is the result of interannual variation in flowering patterns, which is primarily attributed to alterations in local climate or larger meteorological events that affect plant flowering (Elzinga et al., 2007; Frankie et al., 1974; Marquis, 1988; McNeilly & Antonovics, 1968). Thus, the importance of considering several flowering episodes to obtain a better estimation since year-to-year fluctuations may alter the magnitude of this reproductive barrier.

Our results showed that over half of the species-pair comparisons exhibited nearly complete reproductive isolation attributed to non-overlapping flowering phenology. Conversely, the remaining comparisons demonstrated either weak or moderate isolation (RI_F = 0.13–0.56). However, in those instances of incomplete isolation due to temporal reproductive overlap, isolation was subsequently enhanced by a more effective premating barrier linked to floral morphology (discussed further). In two species pairs (*W. nephrolepis*-*W. pedicellata* and *W. nephrolepis*-*W. subsecunda*) almost completely isolated by non-overlapping phenology, the floral morphological barriers were also significant yet redundant.

In a guild of syntopic bat-pollinated bromeliads from the genera *Pitcairnia*, *Pseudalcantarea*, and *Werauhia* in southern Mexico, Aguilar-Rodríguez et al. (2019) documented non-overlapping phenology which apparently prevented interspecific pollen transfer. However, for closely related species growing in sympatry, the flowering time may be constraint by shared ancestry (Rathke & Lacey, 1985). In our case, three species of *Werauhia* bloomed during the dry season and one in the rainy period, while other syntopic species from the study site, *W. notata* and *W. haberi*

(Cascante-Marín et al., 2019; Cascante-Marín et al., 2017), also flowered during the rainy season. The observed variation in flowering patterns suggests an absence of phylogenetic constraint regarding reproductive timing and suggests phenotypic plasticity that may contribute to mitigate reproductive interference.

Mechanical barriers related to flower morphology -- Differences in flower architecture can also prevent gene flow by influencing how pollen is deposited on the pollinator's body (Grant, 1994). Reproductive isolation through morphological differences in flower size plays a major role among some sympatric neotropical groups (i.e., *Achimenes*, Ramírez-Aguirre et al., 2019; *Costus*, Kay, 2006; *Bauhinia*, Albuquerque-Lima et al., 2024). In our study, species conformed into two groups: large (*W. ampla* and *W. nephrolepis*) and small-flowered species (*W. subsecunda* and *W. pedicellata*). The size disparity was nearly 2- to 3- fold between both groups, which suggests that flower-visiting bats would contact the anthers and stigma on different areas of their bodies and prevent interspecific pollen flow. The pollen of the large-flowered *Werauhias* is likely deposited on the bat's head, while for small-flowered species, it is carried on the face (forehead and cheeks) (Fig. 4).

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For species exhibiting comparable floral dimensions and overlapping phenology, such as *W. pedicellata* and *W. subsecunda*, variations in the positioning of anthers and stigma in relation to the corolla mouth are key in preventing interspecific pollen transfer. The lateral positioning of the anthers in *W. subsecunda* flowers likely causes pollen to be deposited on the bat's cheeks, while in *W. pedicellata*, pollen is probably deposited on the top region of the snout and forehead.

Research has shown that in taxonomically unrelated plants pollinated by bats, differential placement of pollen on the pollinator´s body serves as an effective reproductive barrier to interspecific pollination (Muchhala, 2008; Muchhala & Potts, 2007; Steward & Dudash, 2015; Tschapka et al., 2006). Muchhala (2008) proposed that the evolution of this isolation mechanism is particularly facilitated in chiropterophilous (bat-pollinated) plants, attributed to the larger size of bats relative to other pollinator groups, enabling more accurate pollen deposition on specific areas of their bodies.

Our estimations of the strength of mechanical barriers due to floral morphology (RI_{MS} and RI_{MA}) were based on clear-cut and statistically significant differences in size of corolla and reproductive organs (Fig. 3). While differences in stamens position were sufficiently intuitive to assume the existence of a full impediment to interspecific pollen transfer, thus both indices should be interpreted as conservative estimates. Further experimental studies of specific pollen deposition on the pollinator body using pollen dyes or similar techniques may corroborate our interpretation (add references please). However, the high similarity in pollen grain morphology and size overlap among the studied species (unpublished data) precluded any analysis based on detecting differential pollen deposition on stigmas because of unreliable identification.

Interspecific incompatibility and hybrid progeny

The examined postpollination barriers showed a lower strength compared to prepollination barriers, with an average RI value of 0.225 versus 0.614, respectively. The results of the reciprocal crosses indicated that interspecific incompatibility plays an inconsistent role in preventing gene flow among species pairs. In two of the three possible reciprocal crosses, the absence of fruit production reflected a marked incompatibility or incongruity (Vervaeke et al., 2001), although it was not symmetrical in all cases. For example, unilateral incompatibility (Lewis & Crowe, 1958) was observed in crosses between *W. subsecunda* and *W. ampla*, when the former acted as a pollen recipient. However, reciprocal crosses between *W. subsecunda* and *W. pedicellata* revealed partial incompatibility, which allowed the production of hybrid progeny. These permeable postpollination barriers have also been documented among bromeliad species of the genera *Aechmea* (Bromelioideae), *Pitcairnia* (Pitcairnioideae) and *Vriesea* (Tillandsioideae) (Parton et al., 2001; Souza et al., 2017; Wendt et al., 2002). The precise location and mechanisms of operation of this barrier are unknown.

Covas & Schnack (1945) explained the phenomenon of unilateral incompatibility by proposing a positive relationship between pollen size and pistil length in the two species. Stroo (2000), in a compilation of studies on bat-pollinated plants, found a positive correlation between pollen size and stigma length. They suggested that the pollen grain needs to accumulate sufficient resources for tube growth as it traverses the stigma to reach the egg cell (Cruden 2009; Levin, 1971). Moreover, the size and depth of the stigma also play a role, as the pollen grain can draw resources for tube growth from the stylar liquid (Cruden, 2009; Darwin, 1877; Wang et al., 2016). In this context, the larger pollen of *W. ampla* (62–75 µm) successfully reached the ovary of *W. subsecunda*, which has a shorter pistil. Conversely, the smaller pollen of *W. subsecunda* (50–64 µm) was apparently unable to traverse the longer pistil of *W. ampla*. Although this needs confirmation through an analysis of pollen tube growth, this pattern of unilateral incompatibility has also been observed in crosses between congeneric bromeliads of the genera *Aechmea*, *Alcantarea*, and *Vriesea* (Matallana et al., 2016; Souza et al., 2017; Vervaeke et al., 2001).

Further isolation barriers related to seed vigor also showed low effectiveness to prevent gene flow. Although hybrid seeds had some germination capacity, they had a slower germination rate (data not shown). Under natural conditions this slow initial growth of hybrids could mean a higher probability of pathogen attack (Egli et al., 2010) at a plant life stage that is highly vulnerable. In addition, hybrids with lower growth relative to other intra-specific cross plants may later show a disadvantage in the competition for resources (Vaz Mondo et al., 2013) or in their reproduction (Levin, 1971).

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Dio formato: Resaltar

Are there other potential barriers to gene flow among the studied *Werauhia*?

A temporal barrier related to the time of flower anthesis may constitute an additional mechanism of isolation (Levin, 1971). In the studied species, flowers open during the same period in the late afternoon (between 16–17 h) and before the nocturnal pollinator is active (Núñez-Hidalgo & Cascante-Marín, 2024), thus it does not constitute an isolation mechanism. Autogamy or the ability to spontaneously self-fertilize (i.e., selfing) has been proposed by Levin (1971) as a reproductive barrier. For the Bromeliaceae family, Matallana et al. (2016) suggested that selfing was a mechanism to avoid hybridization, due to the high frequency of self-compatibility and autogamy among bromeliads. In a previous study, we found that our studied species showed high levels of autonomous self-fertilization (Núñez-Hidalgo & Cascante-Marín, 2024). This study demonstrated that selfing occurs at the end of the flower's life (i.e., delayed selfing) after the opportunities for cross-pollination have diminished, primarily serving as a reproductive assurance mechanism. Additionally, differences in the number of chromosomes or ploidy levels between species may represent a postmating barrier to prevent the formation of hybrid progeny (Schluter, 2014). Polyploidy has been reported in several groups of bromeliads (Brown & Gilmartin, 1983, 1986; Gitaí et al., 2005; McWilliams, 1974) but basic information on chromosome numbers is lacking for *Werauhia* species in general.

Conclusions

The most significant contribution to total reproductive isolation came from premating barriers, which were on average 2.5 times stronger than postmating barriers (mean RI = 0.614 vs. 0.225, respectively). For half of the species-paired comparisons, non-overlapping flowering schedules alone provided sufficient isolation strength to prevent gene flow (RI_F values > 0.95). When flowering time was insufficient, then differences in floral size and position of reproductive organs in the flower worked in combination to establish a complete reproductive barrier. As a result, the estimates of total reproductive isolation across species pairs were complete (TI = 0.984–1.0; Table 2), suggesting the absence of gene flow between the four Werauhia species studied. When present, postmating barriers were more variable in strength and redundant. Most reproductive barriers were nearly symmetric, which means they exerted comparable strength in both directions between species pairs, except for interspecific compatibility between *W. ampla* and *W. subsecunda*. Our results agree with the general trend described by Christie et al. (2021) but contradict previous suggestions that in the Bromeliaceae family, prepollination reproductive barriers are weak (Matallana et al., 2016; Wendt et al., 2008). Further research involving species pollinated by hummingbirds and bees will enhance our understanding of the reproductive barriers that maintain the local coexistence of highly diverse bromeliad communities. We encourage the use of reproductive isolation indices (RI) to estimate the strength and contribution of the different barriers.

Comentado [D12]: I think it would be appropriate to start this paragraph with something like: Although we did not measure the following parameters in this experiment, we are aware that these may also be important mechanisms to promote isolation and are mentioned below

Comentado [D13]: please explain in a general way what Christie et al. say

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