

Original Article

Repeated evolution of pollination syndromes in a highly diverse bromeliad lineage is correlated with shifts in life form and habitat

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ABSTRACT

Mutualistic plant-pollinator interactions play a critical role in the diversification of flowering plants. The spatiotemporal correlates of such interactions can be understood in a phylogenetic context. Here, we generate ddRAD-seq data for the highly diverse *Vriesea-Stigmatodon* lineage to test for correlated trait evolution among pollination syndromes and life form, habitat type, and altitude. Our results show that pollination syndromes are correlated with changes in life form and habitat type. The ancestor of the *Vriesea-Stigmatodon* lineage was likely bat pollinated, rock dwelling and inhabited open, mid-elevation forests. Transitions from bat to hummingbird pollination are correlated with transitions to the epiphytic life form in shaded habitats, whereas bat pollination is correlated with the rock-dwelling life form and open habitats. Our dated phylogenetic tree reveals independent origins of hummingbird pollination, occurring twice in *Vriesea* at *c.* 5.8 and 5.4 Mya. The timing for the shifts in pollination syndrome coincides with geological and environmental transformations across the Serra do Mar Mountain Chain, which increased habitat heterogeneity where *Vriesea* and their mutualists diversified. The phylogenetic tree reinforces the non-monophyly of taxonomic sections within the genus *Vriesea* previously defined by flower morphology, indicating that some lineages should be treated as species complexes. This study identifies synergetic drivers of speciation in a tropical biodiversity hotspot.

Keywords: Atlantic Forest; bat pollination; ddRAD-seq; hummingbird pollination; Neotropics; phylogenomics

INTRODUCTION

Mutualistic plant-pollinator interactions play a critical role in the diversification of flowering plants, which represent *c.* 90% of the extant plant species diversity on land (Fenster *et al.* 2004,

Crepet and Niklas 2009, WFO 2020). Such interactions have been influenced by abiotic factors (Blois *et al.* 2013, Condamine *et al.* 2018), particularly in the tropics, where high and stable temperature and precipitation levels are favourable to the

formation of diverse mutualistic interactions (Chomicki *et al.* 2019). Shifts in pollination systems can promote plant species diversification, increasing species diversity (van der Niet and Johnson 2012, Givnish *et al.* 2014, Lagomarsino *et al.* 2016, Serrano-Serrano *et al.* 2017). Yet, current knowledge on the spatiotemporal evolution of plant-pollinator interactions and their abiotic correlates remains fragmentary and is mostly lacking for biologically complex ecosystems, such as tropical rainforests.

The study of tropical species-rich plant clades, such as the Neotropical plants bromeliads (Bromeliaceae), can improve our understanding of biotic drivers of diversification. Among the bromeliads, *Vriesea* Lindley is mostly restricted to the Atlantic Forest and to the *campos rupestres* of the Cerrado savanna, two biodiversity hotspots in Brazil (Myers *et al.* 2000, BFG 2018). *Vriesea* includes *c.* 230 species and is especially diverse in the Atlantic Forest, along the Serra do Mar Mountain Chain where *c.* 85% of all species are endemic (BFG 2018, Gouda *et al.* continuously updated). Also, *Stigmatodon* Leme, G. K. Br. & Barfuss was recently segregated from *Vriesea* as an independent genus (Barfuss *et al.* 2016). *Stigmatodon* comprises 20 rupicolous species endemic to the Brazilian inselbergs, occurring exclusively on vertical granite rocky outcrops, with high exposure to solar radiation (Barfuss *et al.* 2016, Couto *et al.* 2022, Gouda *et al.* continuously updated).

Most *Vriesea* species are epiphytes, with the rupicolous (rock dwelling) and terrestrial life forms occurring less frequently (BFG 2018). *Vriesea* occupy different forest strata where they can form either large (more than 100 individuals) or small populations (Costa *et al.* 2014, BFG 2018). *Vriesea* interacts with two main pollinator groups: hummingbirds and bats (Sazima *et al.* 1999, Buzato *et al.* 2000). *Vriesea* species pollinated by hummingbirds have red to yellow floral bracts, tubular flowers with exerted stamens, no scent, and diurnal anthesis (Buzato *et al.* 2000). Conversely, the *Vriesea* flowers pollinated by bats have green to brown floral bracts and campanulate flowers with included stamens, are scented, and have nocturnal anthesis (Sazima *et al.* 1999). The flowers of *Stigmatodon* species are adapted to bat pollination, being similar to the flowers of *Vriesea* with bat-pollination syndrome.

Pollination syndrome consists of a particular set of floral traits, such as shape, colour, scent, and phenology (Faegri and van der Pijl 1979). Syndromes are generally used to infer or predict unobserved pollinators (Fenster *et al.* 2004, Rosas-Guerrero *et al.* 2014, Lagomarsino *et al.* 2017), including in *Vriesea* (Neves *et al.* 2020a). However, this approach has been criticized for oversimplifying complex plant-animal interactions (such as diurnal and nocturnal differences in pollinators; Muchhala 2003) that may lead to unreliable predictions (Ollerton *et al.* 2009, Dellinger 2020).

Avian pollination, epiphytism, and the tank habit (overlapping leaves that accumulate water and organic material) are key innovations in bromeliad species (Givnish *et al.* 2014, Silvestro *et al.* 2014). *Vriesea* occur in habitat types associated with high diversification rates in Bromeliaceae, such as the tropical mountains on which the high diversity has arisen from a combination of factors. Among them, (i) epiphytism allows the plants to occupy a broader range of forest strata,

especially when associated with (ii) the tank habit, which, together with (iii) absorptive trichomes on the leaves, confers independence from soil substrates (Givnish *et al.* 2014). In addition, the association with (iv) different pollinator groups capable of thermoregulation and flying over longer distances (hummingbirds and bats), in contrast to the insects. Finally, humid and steep tropical mountains have (v) abundant rainfall availability throughout the year and (vi) a diversity of habitat types isolated from each other that promotes speciation (Givnish *et al.* 2014).

Differences in both forest habitat types and flight patterns of pollinator groups are used to explain the occurrence of plants in either open or dense vegetation habitats. Bat-pollinated plants often occur exposed in open habitats to facilitate echolocation and view by bats, though they can also occur in dense vegetation when associated with flower scents that guide bats (Muchhala and Serrano 2015). When hovering, bats sweep their wings over a large area around their bodies, whereas hummingbirds are more manoeuvrable, with wing movements restricted to a subtle area directly behind their backs (Muchhala 2003).

Phylogenetic studies within *Vriesea* in the literature have struggled to identify molecular markers capable to discriminate infrageneric groups and delimit species boundaries (Costa *et al.* 2015, Gomes-da-Silva and Souza-Chies 2017, Kessous *et al.* 2020, Machado *et al.* 2020, Loiseau *et al.* 2021). Using the phylogenetic tree from Gomes-da-Silva and Souza-Chies (2017); Kessler *et al.* (2020) assessed the gain and loss of hummingbird pollination in *Vriesea* to illustrate how hummingbirds may lead to increased diversification rates. Kessler *et al.* (2020) identified three shifts from hummingbird to bat pollination in *Vriesea*, but they treated *Stigmatodon* (Barfuss *et al.* 2016) as *Vriesea*. Furthermore, the study identified a gap in our understanding of the correlates of distinct plant-pollinator interactions which would allow for the identification of the drivers of diversification of the clade.

In order to investigate diversification in the *Vriesea-Stigmatodon* clade, we developed novel genomic plastid and nuclear data generated with double digest restriction site associated DNA sequencing (ddRAD-seq; Peterson *et al.* 2012). The use of RAD-seq resolved relationships within species complexes and amongst closely related species with high resolution (Eaton and Ree 2013, Leaché *et al.* 2014, Massatti *et al.* 2016). In bromeliads, RAD-seq data of *Alcantarea* (É.Morren ex Mez) Harms indicated that it is a powerful tool to investigate genetic diversity between closely related species (Lexer *et al.* 2016).

Here, we infer the evolution of plant-pollinator interactions and their morphological and environmental correlates. Specifically, we test the correlated evolution of hummingbird and bat pollination syndromes with life form, habitat type, and altitude through time. We hypothesize that: the correlation with hummingbirds is coupled to the epiphytic life form and occupation of the shaded understory of the Atlantic Forest, whereas bat pollination is associated with the rupicolous and terrestrial life forms in open areas of the forest. Our study highlights the intricate evolution of plant-pollinator interactions and identifies important drivers of tropical biodiversity.

MATERIALS AND METHODS

Sampling

We sampled a total of 59 individual plants, including 47 accessions of *Vriesea* and seven accessions of *Stigmatodon*, with good representation of their morphological variation and geographical distribution (Supporting Information, Material S1). We sampled around 20% of the hyperdiverse *Vriesea* (with a total of 230 species, Gouda *et al.* continuously updated). Difficulty in extracting high-quality DNA, which is required for Next Generation Sequencing techniques like ddRAD-seq, hampered a more complete sampling. *Vriesea* was formerly treated as a single genus (Smith and Doowns 1977), but was segregated into seven genera distributed in two different subtribes (Cipuropsidinae and Vrieseinae) consisting the tribe Vrieseae (Grant 1995, Barfuss *et al.* 2016, Leme *et al.* 2017). Species from the related genera *Alcantarea* (subtribe Vrieseinae), *Lutheria* Barfuss & W.Till, *Goudaea* W.Till & Barfuss (subtribe Cipuropsidinae), and *Tillandsia* L. (tribe Tillandsieae, after Barfuss *et al.* 2016) were included as outgroups. We collected samples from natural populations mainly in the Atlantic Forest and Cerrado, prioritizing type localities. Additional samples were collected from living collections. Information on vouchers is presented in Supporting Information, Material S1. Tillandsioideae species are mostly diploid $2n = 50$, as shown by previous studies (Cotias-de-Oliveira *et al.* 2004, Palma-Silva *et al.* 2004, Ceita *et al.* 2008, Manhães 2021).

DNA extraction, library preparation, and sequencing

We extracted DNA from silica gel-dried leaf material and then stored it at -80°C . Frozen leaf samples were crushed into powder using a TissueLyser II (Qiagen). We extracted DNA using the CTAB protocol of Doyle and Doyle (1987), with modifications following Azmat *et al.* (2012). We determined DNA extraction quality on a 1% agarose gel and quantified DNA using a NanoDrop[®] spectrophotometer and a Qubit[®] fluorimeter 3.0 (High sensitivity kit; Life Technologies).

We standardized DNA samples to concentrations of 10 ng/ μl and a total of 50 μl of each sample. Library preparation and single-end DNA sequencing on an Illumina HiSeq 2000 was performed by Floragenex Inc. (Eugene, OR, USA). Total DNA was double-digested with the *SbfI* and *PstI* enzymes (ddRAD-Seq, Peterson *et al.* 2012).

Genome assembly and mapping

We performed *de novo* assembly using the software pipeline PyRAD v.3.0.4 (Eaton 2014). Genomic data for each species varied and PyRAD was used to assemble loci by optimizing coverage across datasets. Optimization through an alignment-clustering algorithm allowed for indel variation within and between samples, recovering more shared loci across disparate taxa (Eaton 2014). We defined the following parameters: minimum coverage per cluster = 6, clustering threshold = 0.85, minimum sample coverage for loci = 40, maximum number of individuals with shared heterozygous sites = 3, and the remaining parameters were set to default.

To identify the chloroplast loci in our dataset, we performed a Bowtie search against the pineapple chloroplast genome (GenBank accession number NC_026220.1), using Bowtie2

with the ‘-very-sensitive-local’ parameter (Langmead and Salzberg 2012).

Phylogenetic analyses

Phylogenetic trees were inferred using Maximum Likelihood (ML) and coalescent approaches. ML based on the concatenated nuclear and plastid loci was inferred using RAXML-HPC2 on XSEDE via the CIPRES Science Gateway v.3.3 (Miller *et al.* 2010), setting a GTR+GAMMA substitution model and rapid bootstrap (BS) estimation based on 1000 replicates. We interpreted BS values ≥ 90 as strong, 89–70 as moderate, and 69–50 as weak support (Hillis and Bull 1993). We also ran ML using the PhyML online platform v.3.0 (<http://www.atgc-montpellier.fr/phyml/>) with the following parameters: automatic model selection-AIC, tree searching nearest neighbour interchange, and a branch support approximate likelihood-ratio test Shimodaira–Hasegawa-like (aLRT SH), which is a simpler and faster branch support test recommended for large molecular datasets (Guindon *et al.* 2010).

Coalescent analyses were performed for the nuclear loci using ASTRAL-III (Zhang *et al.* 2018) and SVDquartets (Chifman and Kubatko 2014), following Ferreira *et al.* (2022). Briefly, ASTRAL-III was ran based on the unrooted trees estimated by ML searches in RAXML (Stamatakis 2014) and branch support was evaluated using local posterior probabilities (PP). SVDquartets was inferred with exhaustive sampling all possible quartets. Branch support was evaluated using 1000 non-parametric BS replicates.

We estimated the divergence times using a penalized likelihood approach in treePL (Smith and O’Meara 2012). We used the RAXML tree with the concatenated dataset as input and secondary calibrations at the Vrieseinae crown node (5.4–10.2 Mya) and at the *Vriesea* crown node (3.3–6.8 Mya) based on Givnish *et al.* (2014) and Kessous *et al.* (2020). To calculate the 95% confidence interval of node ages, we ran treePL on each of the BS trees from RAXML, then used TreeAnnotator from BEAST v.1.10.4 (Suchard *et al.* 2018) to generate a summary tree. We visualized phylogenetic trees of all methods described above using FigTree v.1.43 (Rambaut 2014).

Ancestral trait reconstruction

We estimated ancestral states for pollination syndrome (hummingbird and bat), life form (epiphyte, terrestrial, and rupicolous), habitat type (open and shade), and altitude (continuous values) to understand when and how many times these traits evolved in *Vriesea* and *Stigmatodon*. Information on traits was extracted from field observations, monographs, floras, and recent taxonomic reviews (Supporting Information, Material S2; Smith and Downs 1977, Versieux and Wanderley 2008, Costa *et al.* 2009, Moura, 2011, Nogueira 2013, Neves *et al.* 2018, 2020a, Uribbe *et al.* 2020, Couto *et al.* 2022). We used the dated tree from treePL built with the concatenated dataset for character optimizations. For ancestral states reconstruction of discrete traits, we used Bayesian stochastic character mapping (Bollback 2006) estimated from 1000 iterations with the function *make.simmap* in the R package *phytools* (Revell 2012, R Core Team 2018). We coded pollination syndromes based on flower and floral bract traits, following Neves *et al.* (2020a), who validated

the utility of pollination syndromes in *Vriesea* based on information on confirmed pollinators from the literature. There are some *Vriesea* species known to be visited and/or pollinated by both hummingbirds and bats (Sazima et al. 1995, Aguilar-Rodríguez et al. 2019). These studies show that bat-pollinated flowers can be visited by hummingbirds at dawn, but they feed on the small amount of nectar left by the bats in withered flowers. In such cases, based on floral syndromes, we coded one group as the primary pollinator, considering that pollinators efficacy and efficiency was not tested in these studies. For the continuous trait of altitude, we used a ML reconstruction with the function *contMap* from *phytools*. We used the mean altitude for each species based on information extracted from the dataset compiled in Ramos et al. (2019) for Atlantic Forest epiphytes, complemented with data from our personal collections (Supporting Information, Material S2).

Correlation among traits

In order to test the correlation of pollination syndrome with life form, habitat type, and altitude, we fitted Bayesian threshold models (Felsenstein 2012, Revell 2012) using the function *threshBayes* in *phytools*. We modelled pollination syndrome, life form, and habitat type as discrete binary traits and altitude as a continuous trait. We divided the multistate trait life form into epiphyte or terrestrial/rupicolous. When coding life form, we considered the predominant state for polymorphic species. In addition, merging terrestrial and rupicolous states, in this specific case, brings greater statistical power to our analysis, as we use fewer parameters. The great majority of *Vriesea* are epiphytes (c. 150 spp.) and the terrestrial and rupicolous *Vriesea* are the minority (BFG 2018). We ran the Markov chain Monte Carlo (MCMC) for 3 million generations sampling every 1000, with a burn-in of 20% to summarize the posterior distribution values for the correlation coefficient (r). We calculated the effective sample size (ESS) of the coefficient using the function *effectiveSize* in the R package *coda* (Plummer et al. 2006).

Distribution patterns of hummingbird and bat pollination syndromes

In order to infer distribution patterns of species from hummingbird and bat pollination syndromes, we built spatial plots of altitude vs. latitude. With this analysis we aim to detect if plant species from hummingbird and bat pollination syndromes are spatially segregated or not. Because distribution patterns are likely correlated to preferences of habitat, physiology, and other ecological aspects of their specific pollinator species (Aguilar-Rodríguez et al. 2019, Kessler et al. 2020), we used data from the literature on documented *Vriesea* pollinator interactions to build a plant–pollinator network. Taken together, this approach allowed a better visualization and an integrated discussion of *Vriesea* spatial distribution and pollinator-specific interactions. There is a complete lack of pollination studies for *Stigmatodon*, so it was not included in this analysis. Further, the current 20 *Stigmatodon* species show a clear bat pollination syndrome and their trait and spatial variation is captured within the variation in *Vriesea* (Barfuss et al. 2016, Couto et al. 2022).

To map spatial distribution, we built plots using 9568 occurrence records for 132 species from the dataset of Ramos et al.

(2019). First, we generated boxplots for each species using altitudinal data and excluded the outliers using the function *boxplot* in the R package *graphics* (Murrell 2018). Then, we used *ggplot2* (Wickham 2011) to produce the altitude vs. latitude plots for species of each syndrome.

In addition, we used the data on pollination biology compiled by Neves et al. (2020a), including all reported *Vriesea* pollinator interactions in both peer-reviewed and grey literature. We extracted information on the identity of plants and floral visitors and pollinators. In total, we documented interactions between 35 *Vriesea* species and 16 hummingbird and three bat species (Supporting Information, Material S3). The most representative hummingbird pollinators are *Phaethornis eurynome* Lesson, *Ramphodon naevius* Dumont, *Thalurania glaucopis* Gmelin, and *Leucochloris albicollis* Vieillot, interacting with 19, 12, 11, and nine *Vriesea* species respectively. The bat species *Anoura caudifer* É. Geoffroy is recorded visiting flowers of each of the eight registered *Vriesea* species with bat pollination syndrome. We used this information to interpret and discuss our results.

RESULTS

Genome assembly and mapping

We generated in total 85 GB of raw data containing 358 425 268 reads of 100 bp each. The final dataset comprised 664 ddRAD-seq loci, 11 of which were from the chloroplast genome. The concatenated alignment totalled 57 640 bp in length, with 4878 variable sites, 1868 of which were parsimony informative sites. The percentage of gaps and missing data was 21.99%.

Phylogenetic relationships and clade age

The ML tree resolved high support for most of the deep phylogenetic relationships (Fig. 1). Subtribe Vrieseinae (BS = 97, aLRT SH = 1) and each genus were monophyletic, including *Stigmatodon* (clade A, BS = 98, aLRT SH = 1) and *Vriesea* (clade B, BS = 93, aLRT SH = 1). We identified two distinct lineages of both hummingbird- and bat-pollinated species in *Vriesea*, emerging from two main clades: C (BS = 87, aLRT SH = 99) and D (BS = 54, aLRT SH = 28). Clades E (BS = 23, aLRT SH = 99) and F (BS = 65, aLRT SH = 99) are two large lineages of species exclusively from each of the two pollination syndromes (Fig. 1). We recovered poor resolution for shallow nodes; however, some groupings of morphologically similar species gained high support. In other cases, different accessions of the same species were not resolved as monophyletic, such as *Vriesea agostiniana* E. Pereira. The topology shown here refers to the ML tree inferred in RAxML, which presented few incongruences in the shallow relationships within *Vriesea* when compared with the ML tree inferred in PhyML and the coalescent trees inferred with both Astral and SVDquartets (Supporting Information, Materials S4, S5). These incongruences are mostly at poorly supported nodes.

We estimated the crown age of subtribe Vrieseinae in the Late Miocene 10.1 million years ago (Mya) [95% High Posterior Density (HPD): 10.18–10.19 Mya], *Stigmatodon* 8.0 Mya (95% HPD: 7.4–8.6 Mya), and *Vriesea* 6.3 Mya (95% HPD: 5.5–6.7 Mya). All shifts among syndromes in *Vriesea* occurred between 5.8 and 5.4 Mya (95% HPD: 4.4–6.2 Mya; Fig. 2).

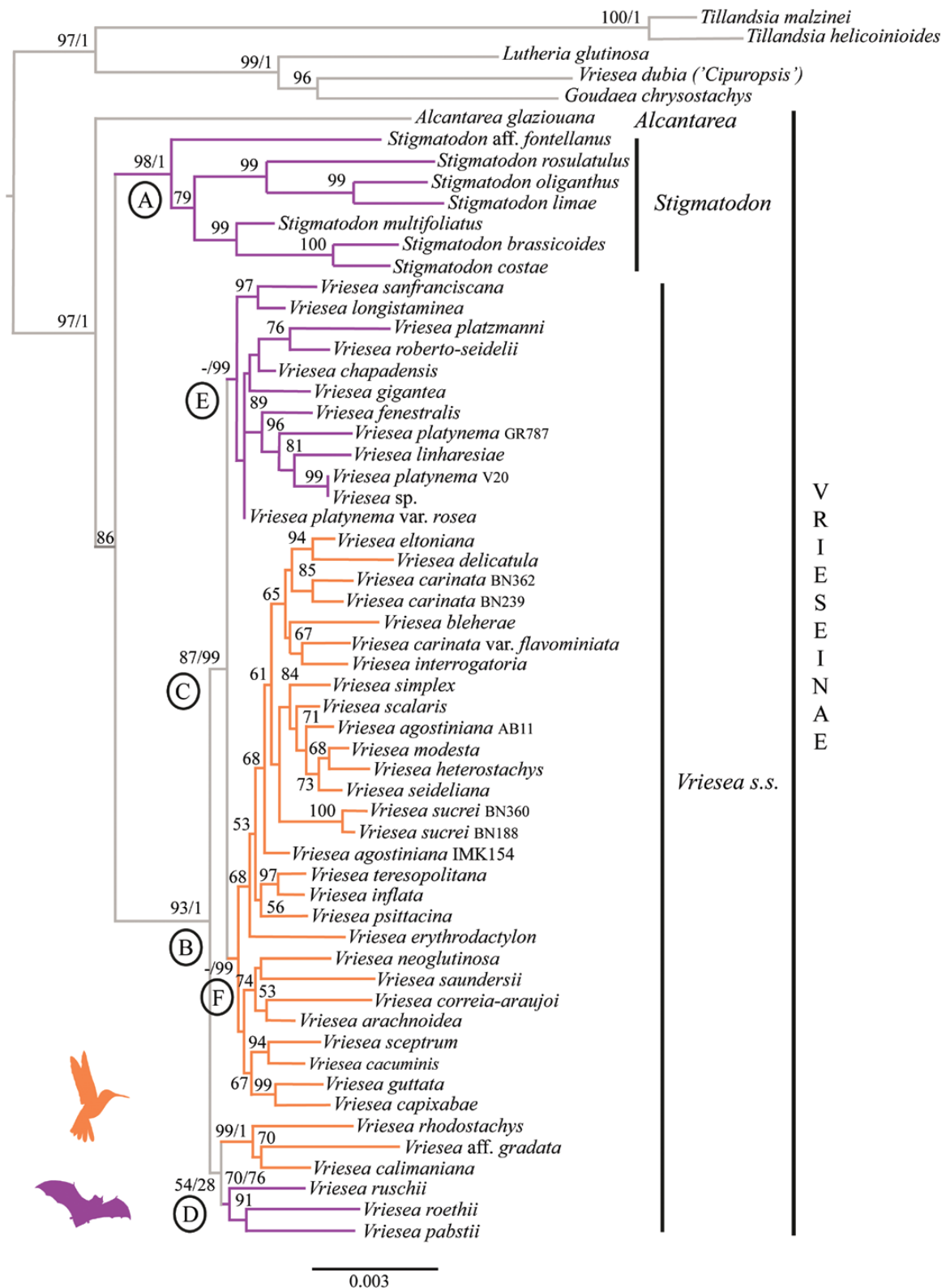


Figure 1. ML tree of *Vriesea* and *Stigmatodon* based on 664 ddRAD-seq loci showing hummingbird-pollinated and bat-pollinated lineages. BS support values above 50% are shown at internodes. For selected nodes discussed in the text, we show approximate likelihood-ratio test Shimodaira–Hasegawa-like (aLRT SH-like) support. Pollination syndromes are shown to evolve repeatedly in *Vriesea*.

Ancestral trait reconstruction and evolutionary correlates of pollination syndrome

For *Vriesea*, we inferred a bat pollination syndrome as the ancestral state (PP = 0.95; Fig. 3A), and two independent shifts to

hummingbird pollination. For habitat, the ancestral condition was inferred to be shaded environments (PP = 0.99; Fig. 3B) and we recovered seven independent transitions to open areas. The epiphytic life form was ancestral (PP = 0.99; Fig. 3C), with

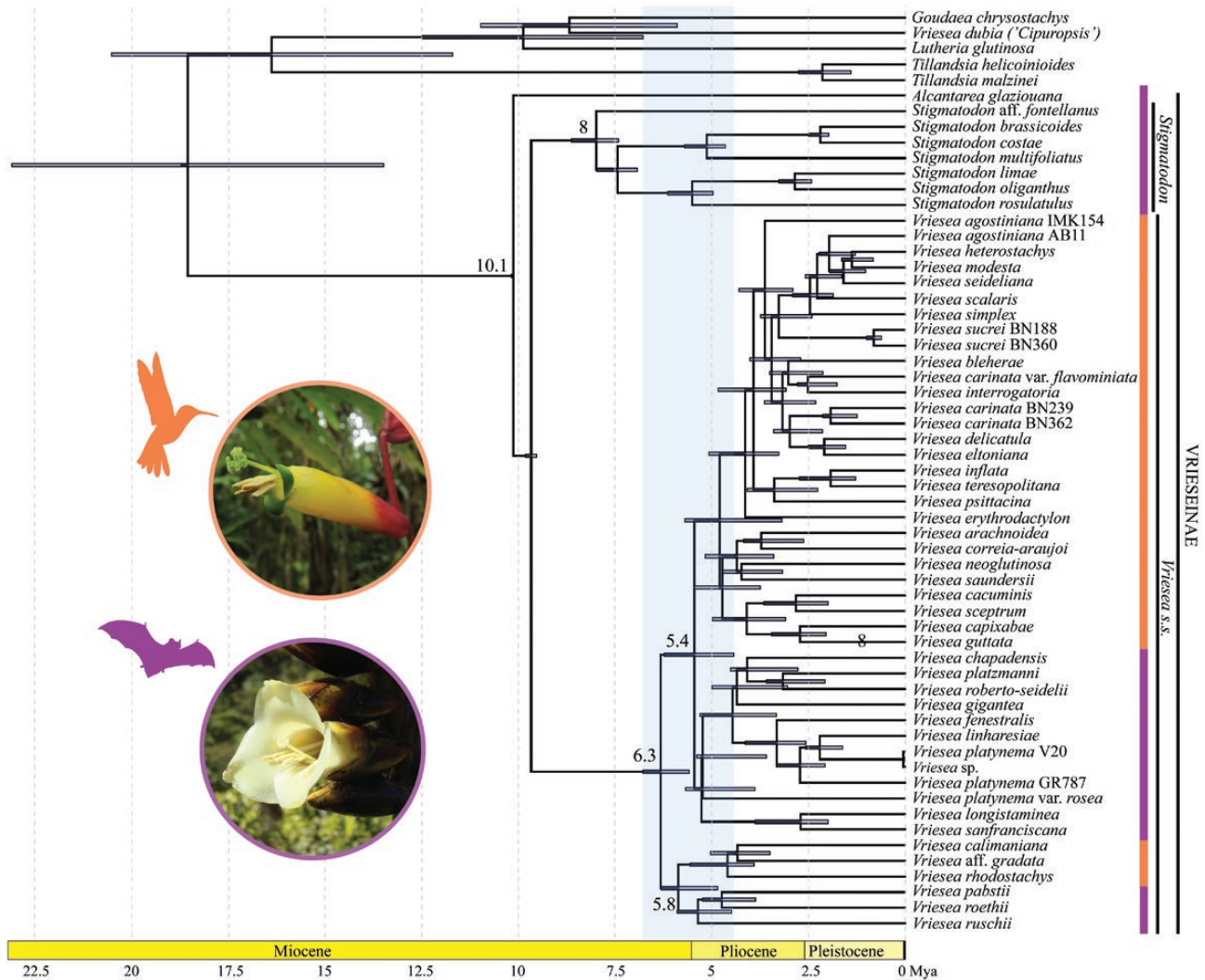


Figure 2. Dated phylogenetic tree for *Vriesea* and *Stigmatodon* based on 664 ddRAD-seq loci estimated in treePL. Node bars indicate 95% HPD for the age of each node. The Holocene is indicated with a bold black line. The root age of *Vriesea* is estimated to be placed in the Late Miocene (6.3 Mya) and the repeated shifts between pollination syndromes are estimated to occur in the Late Miocene and Early Pliocene (5.8–5.4 Mya; blue shading indicates 95% HPD of timing of the shifts). The tectonic events occurring at that time, which re-shaped the scarp of the Serra do Mar Mountain Chain, likely increased habitat heterogeneity in which plant lineages and their mutualists diversified.

the terrestrial and rupicolous life forms evolving multiple times in this hyperdiverse genus (at least 12 and 10 times, respectively). For *Stigmatodon*, we recovered bat pollination syndrome (PP = 1.00; Fig. 3A), open environments (PP = 0.94; Fig. 3B), and rupicolous life form (PP = 0.97; Fig. 3C) as ancestral states. For the larger clade *Vriesea-Stigmatodon* we recovered bat pollination syndrome (PP = 0.97; Fig. 3A), open environments (PP = 0.63 Fig. 3B), and rupicolous life form (PP = 0.67; Fig. 3C) as ancestral states.

When testing for phylogenetic correlation, the strongest effect was the relationship between pollination syndrome and habitat type ($r = 0.39$, 95% HPD -0.03 to 0.79). We also found a comparatively weaker relationship between pollination syndrome and life form (Supporting Information, Material S6; $r = -0.29$, 95% HPD -0.66 to 0.12), representing a moderate correlation overall. We inferred that *Vriesea* ancestors, as well as *Stigmatodon* and *Vriesea-Stigmatodon* clade ones, occupied mid-elevations (c. 700 m a.s.l.), with at least eight transitions to highlands and

ten to lowlands in *Vriesea* (Supporting Information, Material S7). No correlation among pollination syndromes and altitudinal distribution was found ($r = -0.07$, 95% HPD -0.40 to 0.26, Supporting Information, Material S7).

Distribution patterns of hummingbird and bat pollination syndromes

Vriesea species are widely distributed throughout the latitudinal and altitudinal range of the Atlantic Forest, with some of them reaching the savanna (Brazilian Cerrado domain, in the high *campos rupestres*) (Supporting Information, Material S8). Based on our dataset, *Vriesea* is documented from sea level to 2162 m, between latitudes 3°S to 32°S (except for 10% of the species occurring in the Andes, Amazon, and Greater Antilles; Barfuss et al. 2016). High species richness is found in altitudes up to 1200 m and between latitudes 15°S to 27°S. We found no difference between the occupation of latitudinal and altitudinal space between species with hummingbird and bat pollination

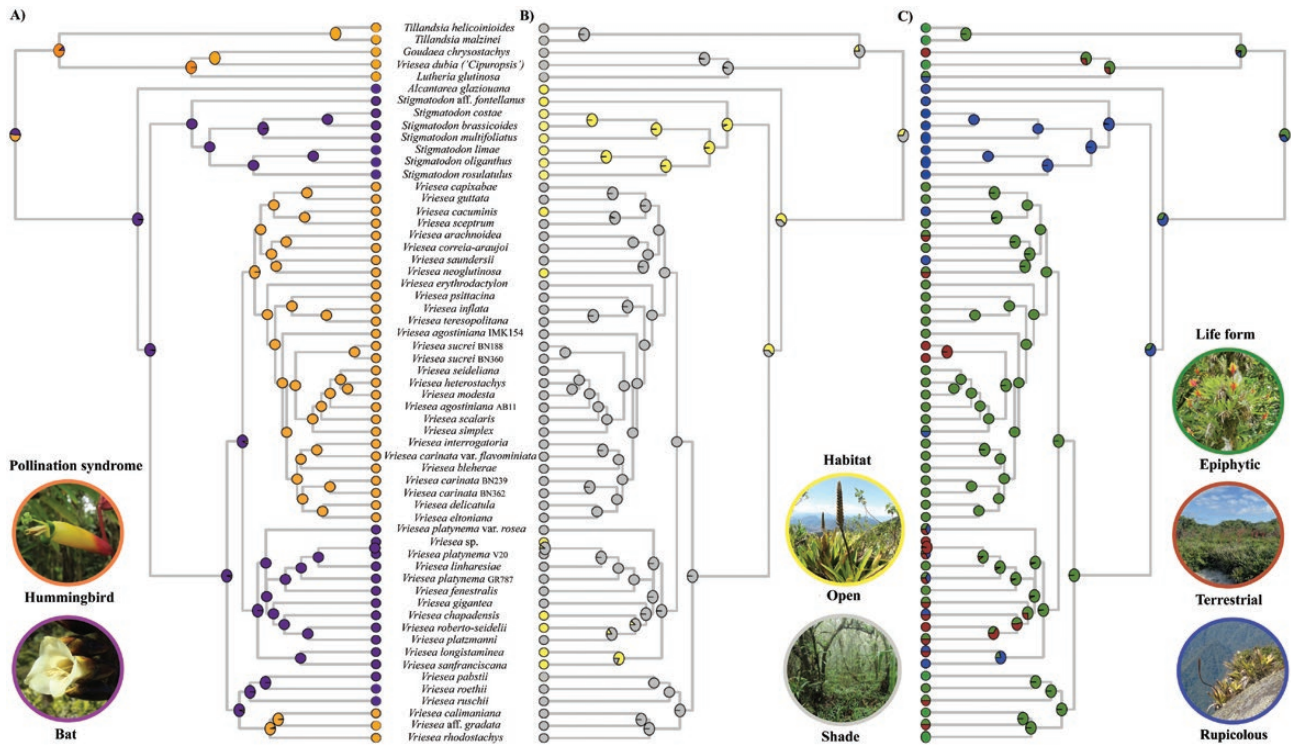


Figure 3. Ancestral trait estimation for (A) pollination syndromes, (B) habitat types, and (C) life forms of *Vriesea* and *Stigmatodon*. Pie charts at nodes represent posterior probabilities of ancestral states using Bayesian inference. The most recent common ancestor of *Vriesea* is inferred to have been a bat-pollinated, epiphytic plant growing in shaded forests. Shifts in pollination syndrome are correlated with shifts in habitat type and life form.

syndromes. Instead, our results indicate that species associated with bats reach the highest altitudes (Supporting Information, Material S8).

DISCUSSION

The phylogenetic tree of *Vriesea* and its sister group *Stigmatodon* using ddRAD-seq data revealed two independent origins of hummingbird pollination syndrome (Fig. 1). We infer *Vriesea* to have originated at 6.3 Mya and its ancestor to be a bat-pollinated epiphyte distributed in shaded, mid-elevation areas of the Atlantic Forest; and *Stigmatodon* to have originated at 8 Mya and its ancestor to be a bat-pollinated, rupicolous species found in open, mid-elevation granite rocky outcrops (Fig. 3; Supporting Information, Material S7).

We have corroborated our hypotheses by showing that pollination syndrome likely evolved jointly with life form and habitat type (Supporting Information, Material S6). The inferred shifts from bat to hummingbird pollination correlated with shifts to epiphytism and shaded habitat, whereas bat-pollination correlated with the rupicolous and terrestrial life forms in open areas. Shifts among syndromes were inferred at around 5.8–5.4 Mya, during the Late Miocene and Early Pliocene (Fig. 2). At that time, tectonic events led to geological and environmental transformations in the Serra do Mar Mountain Chain, likely resulting in an increased habitat heterogeneity in which plant lineages and their mutualists diversified (Almeida 1976, Azevedo *et al.* 2020, Neves *et al.* 2020b). Additionally, when investigating distribution

patterns, we showed *Vriesea* species from both syndromes are distributed across wide latitudinal and altitudinal ranges of the Atlantic Forest, reaching part of the Cerrado savanna (Supporting Information, Material S8). To further explore this broad occurrence pattern, we leveraged *Vriesea* pollinator interactions from compiled literature records to discuss the influence of pollinator variety on ecological preference (Supporting Information, Material S8).

Pollination syndromes evolve in correlation with life form and habitat type

We inferred a bat pollination syndrome, epiphytic life form, and shaded habitat as the ancestral states of *Vriesea*, and bat-pollinated, rupicolous life form and open habitat as ancestral states of *Stigmatodon* (Fig. 3). Moreover, we showed that transitions from bat to hummingbird pollination were often linked to transitions to the epiphytic life form in shaded habitats, whereas bat pollination was linked to the rupicolous life form and open habitats. Givnish *et al.* (2014) inferred the association between fertile and humid mountains with epiphytism in Bromeliaceae, suggesting that in tropical forests, abundant rainfall and the nutrient-rich release of organic material from both plants and animals explain the great richness of epiphytic species. Givnish *et al.* (2014) also suggested such habitats favour avian pollination, especially by hummingbirds, as cool and wet conditions select for thermoregulating pollinators (Cruden 1972, Kessler *et al.* 2020). The genus *Vriesea* presents all these characteristics, allowing for an understanding of the evolutionary correlates of different pollination syndromes.

Despite being distributed across the entire altitudinal and latitudinal range of the Atlantic Forest (Supporting Information, Material S8), we identified a broad occurrence pattern for *Vriesea* species: hummingbird-pollinated species are often less exposed in shaded habitats and bat-pollinated species are often more exposed in open habitats. Species with flowers adapted to hummingbirds are predominant in *Vriesea* (c. 137 out of 230 spp.). They are mostly epiphytes concentrated in the understory at mid elevation, but also occur on coastal plains (*restingas*) and reach high fields and rocky outcrops (*campos de altitude* and *campos rupestres*), as terrestrial or rupicolous species and rarely epiphytes (BFG 2018). The main hummingbird species recorded pollinating *Vriesea* are the hermits of the subfamily Phaetornithinae (*P. eurynome* and *R. naevius*, Neves *et al.* 2020a; Supporting Information, Material S4). The hermits commonly inhabit the understory of Neotropical forests and their diversity decreases at high elevations and in dry habitats (Rodríguez-Flores *et al.* 2019; Supporting Information, Material S9). In the Atlantic Forest, *R. naevius* is amongst the main pollinators in humid lowlands up to 500 m, while *P. eurynome* is predominant at higher altitudes, around 1500 m (Buzato *et al.* 2000, Vizentin-Bugoni Maruyama and Sazima 2014; Supporting Information, Material S9). The non-hermit *Vriesea* pollinators of the subfamily Trochilinae (*Thalurania glaucopsis*, *Leucochloris albicollis*, *Florisuga fusca* Vieillot and *Amazilia fimbriata* Gmelin) most commonly forage in forest canopies (Supporting Information, Material S9).

Vriesea species with flowers adapted to bats are generally associated with the forest canopy, occurring as epiphytes from low- to highlands. In the *campos de altitude* and *campos rupestres* open fields, they usually occur as rupicolous or terrestrial species. Due to their small size and high metabolism, nectar-feeding bats need to quickly locate the flowers to feed on using olfaction, vision, and echolocation (Helversen and Winter 2003). An experimental study conducted with the two main bat pollinator species for *Vriesea* (*A. caudifer* and *Anoura geoffroyi* Gray) showed that well-exposed flowers facilitate echolocation and vision, while in a dense forest matrix, bats are more dependent on flower scent and are guided by olfaction (Muchhala and Serrano 2015). These two *Anoura* species inhabit primary and secondary forests in Brazil, reaching altitudes up to 2000 m (Supporting Information, Material S9). Accessibility of flowers is shown to affect bat pollination in *Burmeistera* H.Karst. & Triana species, where more exposed flowers have an increase in nocturnal pollen deposition (Muchhala 2003). Differences in flight patterns of the two pollinator groups in *Burmeistera* could be an explanation of the occurrence of plants in open or dense vegetation habitats, as bats sweep their wings over a large area around their bodies while hovering, while hummingbirds are more manoeuvrable, with wing movements restricted to an area directly behind their back. Here, we suggest a similar function in *Vriesea*, where pollinators exert selective pressure(s) on plant habitat. Taken together, these lines of evidence support the main occurrence of *Vriesea* hummingbird-pollinated species in shaded and dense vegetation, and the concentration of *Vriesea* bat-pollinated plants in open and exposed habitats.

We inferred the shifts among syndromes to have happened around 5.8–5.4 Mya (Fig. 2), coinciding with tectonic events of the Late Miocene and Early Pliocene that continued shaping the

Serra do Mar Mountain Chain in the Atlantic Forest (Almeida 1976, Turchetto-Zolet *et al.* 2013, Guedes *et al.* 2020). Such tectonic events promoted orogenic transformations likely resulting in habitat barriers in which plant species and their mutualists diversified. Likewise, other Atlantic Forest lineages are hypothesized to have diverged during the same period and to be influenced by such changes (Grazziotin *et al.* 2006, Fitzpatrick *et al.* 2009, Thomé *et al.* 2010). Here, we used a penalized likelihood method to estimate divergence times for *Vriesea* instead of a Bayesian approach as in Kessous *et al.* (2020), which is fast and suitable for analysis of large datasets. However, our approach does not account for fossil and branch length uncertainty explicitly (e.g. Reis *et al.* 2016). Our methodological approach in addition to the distinct sources of information for secondary calibrations and the inclusion of the *Vriesea limae* L.B.Sm.clade as *Stigmatodon* (Couto *et al.* 2022), explain the differences in divergence times compared to Kessous *et al.* (2020) and Loiseau *et al.* (2021). In general, our topology is congruent with other published phylogenies for the study group, especially for deep nodes (Kessous *et al.* 2020, Machado *et al.* 2020, Loiseau *et al.* 2021).

The specific habitat zones where the shifts between pollination syndromes may occur are hypothesized by Kessler *et al.* (2020) to be those where changes in pollinator's physiological preferences occur, at mid elevations and in the transitions between humid and dry areas. This hypothesis is based on a widely recognized distribution pattern of hummingbird-pollinated species being more diverse at cool, wet, and mid to high elevations, whereas bat-pollinated species are more diverse in humid, mid to low elevations (Kessler *et al.* 2020). However, this pattern is recognized in studies developed along wide altitudinal ranges, such as along the Andean slopes that reach altitudes of more than 4000 m. In contrast, our study region—the Atlantic Forest—only reaches altitudes of c. 2200 m, showing higher species diversity of both hummingbird- and bat-pollinated plant assemblages and their pollinators in the lowlands, with a decrease of diversity towards the highlands (Sazima *et al.* 1999, Buzato *et al.* 2000).

Species with intermediate floral morphology among the two pollination syndromes in *Vriesea* are hypothesized to be a product of pollinator shifts (Neves *et al.* 2020a). These intermediate species occur across the entire altitudinal range of the genus, both in dry and wet habitats. In mutualisms that span environmental gradients, specific interactions can change with biotic and abiotic variables such as regional species guild, temperature, light, and precipitation (Chomicki *et al.* 2019). Broadly, we show life form and habitat type to influence plant–pollinator interactions. Although we cannot rule out the hypothesis of no correlation among such traits (considering the 95% posterior distribution of correlation coefficients, Supporting Information, Material S6), we present corroborating evidence from field studies. Studies at the community level would further clarify these factors shaping the distribution patterns of pollination syndromes.

Repeated evolution of pollination syndromes and taxonomic implications

We identified two independent lineages of hummingbird-pollinated species in *Vriesea* (Figs 1, 3A), resulting in the repeated

evolution of pollination syndromes. Such findings reinforce the non-monophyly of *Vriesea* sections (*V.* section *Vriesea* and *V.* section *Xiphion*) that were defined based on morphological traits that reflect the pollination syndromes [Smith and Downs 1977](#).

Within each pollination syndrome, we recovered well-supported clades of morphologically similar species ([Fig. 1](#)). Among the hummingbird pollination syndrome are: *Vriesea teresopolitana* Leme + *Vriesea inflata* (Wawra) Wawra both from the *V. inflata* group, which present simple inflorescences with congested and inflated floral bracts ([Costa et al. 2009](#), [Gomes-da-Silva and Souza-Chies 2017](#)); *Vriesea guttata* Linden & André and *Vriesea capixabae* Leme, with pendulous inflorescences and roseous bracts fully covered with a white-waxy indument ([Leme 1999](#)); *Vriesea rhodostachys* L.B.Sm., *Vriesea* aff. *gradata* (Baker) Mez, and *Vriesea calimanihana* Leme & W.Till, presenting robust simple inflorescences with large, cartaceous, very inflated, and imbricate floral bracts ([Leme et al. 1997](#)); and *Vriesea sceptrum* Mez and *Vriesea cacuminis* L.B.Sm., both with tubular yellow flowers with included stamens, which differ from the typical hummingbird-pollinated flowers in the genus. Among the bat pollination syndrome are: *Vriesea platynema* Gaudich. + *Vriesea linharesiae* Leme & J.A.Siqueira + *Vriesea* sp. with included stamens, leaves green with a purple reddish macule in the apex ([Leme and Siqueira-Filho 2001](#), [Moura 2011](#)); *Vriesea roethii* W.Weber and *Vriesea pabstii* McWill. & L.B.Sm., with mostly green inflorescences and white flowers with included stamens ([Smith and Downs 1977](#), [Weber 1979](#)); and *Vriesea sanfranciscana* Versieux & Wand. and *Vriesea longistaminea* C.C.Paula & Leme, with exerted and slightly spread long stamens, which differ from the typical bat-pollinated flowers in the genus ([Moura 2011](#)).

Despite shared morphology and, in some cases, geographic distribution, these groupings are not exclusive for each species complex or morphological group. Further, different accessions of the same nominal species can be spread across the phylogenetic tree, rather than forming a monophyletic group ([Fig. 1](#)). In addition to the poor clade resolution—resulting from the difficulty in finding informative molecular markers for *Vriesea*, such findings are suggestive of incomplete lineage sorting with retention of ancestral polymorphism and incipient speciation ([Goetze et al. 2017](#)). The genus is relatively young (crown age 6.3 Mya) and species may have not experienced sufficient trait or genetic coalescence. Hybridization cannot be discarded as an alternative possible explanation, as it has been shown to occur within *Vriesea* and between *Vriesea* and other genera ([Matos et al. 2016](#), [Zanella et al. 2016](#), [Neri et al. 2017](#), [Loiseau et al. 2021](#)). These studies identified breaks in reproductive isolation, such as overlapping flowering times and shared pollinators, as the main drivers of hybridization in the group. A large phylogenetic tree of *Vriesea* based on plastome data revealed similar results regarding the non-monophyly of species ([Machado et al. 2020](#)).

Recent studies identified repeated evolution of pollination syndromes occurring broadly in Bromeliaceae ([Givnish et al. 2014](#), [Aguilar-Rodríguez et al. 2019](#)) and in other diverse tropical groups such as the Gesneriaceae and Campanulaceae ([Lagomarsino et al. 2017](#), [Serrano-Serrano et al. 2017](#)), Acanthaceae ([Tripp and Manos 2008](#)), and Passifloraceae ([Abrahamczyk et al. 2014](#)). We found two shifts from bat to

hummingbird pollination ([Fig. 3A](#)), whereas the reverse pattern (from hummingbird to bat pollination) is more frequently documented across angiosperms. Our findings reject the hypothesis that bat pollination is an evolutionary dead end ([Tripp and Manos 2008](#), [Fleming et al. 2009](#)) and corroborates evidence of high transition rates from bat to hummingbird pollination ([Lagomarsino et al. 2017](#)). Although [Kessler et al. \(2020\)](#) recovered hummingbird pollination as the ancestral state of *Vriesea*, with three shifts from hummingbird to bat pollination, this is due to their inclusion of bat-pollinated *Stigmatodon* ([Barfuss et al. 2016](#)) as *Vriesea*. We here follow the classification of [Barfuss et al. \(2016\)](#) in accepting *Stigmatodon* as monophyletic, which is supported by the comprehensive genomic data presented here and in [Leme et al. \(2017\)](#), [Kessous et al. \(2020\)](#), [Machado et al. \(2020\)](#), and [Loiseau et al. \(2021\)](#).

CONCLUSION

Here we identified evolutionary correlates of plant–pollinator interactions in an ecologically and morphologically diverse Neotropical plant clade. Our results indicate that pollination syndromes evolved in association with shifts in plant life form and habitat type in *Vriesea* and its sister group *Stigmatodon*. We identified a broad pattern of occurrence of hummingbird-pollinated species in shaded and dense-forested areas, and bat-pollinated plants in open and more exposed habitats. We inferred bat pollination, epiphytic life form, and occupancy in shaded and mid-elevation habitats as ancestral states in *Vriesea*. The repeated evolution of pollination syndromes explains the non-monophyly of the two *Vriesea* sections defined on flower morphology. As biodiversity loss intensifies globally ([WWF 2020](#)), it is crucial to understand the relationship between plants and their pollinators to avoid erosion of the complex ecological networks of tropical ecosystems and their capacity for maintenance and continued evolution.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Material S1. Taxa included in the phylogenetic analyses with their respective vouchers and information on locality and origin of the samples.

Material S2. Traits used for the ancestral character reconstruction and correlation analyses.

Material S3. Data on pollination biology compiled by [Neves et al. \(2020a\)](#).

Material S4. The full annotated ML trees of *Vriesea* and *Stigmatodon* based on 664 ddRAD-seq loci inferred with both (A) RAxML with BS support and (B) PhyML with aLRT SH-like support.

Material S5. The full annotated coalescent trees of *Vriesea* and *Stigmatodon* based on nuclear ddRAD-seq loci inferred with both (A) Astral with posterior probability and (B) SVDquartets with BS support.

Material S6. Density plot of the posterior distribution of the correlation coefficient of the Bayesian threshold models built to test association of pollination syndromes with (A) habitat type, (B) life form, and (C) altitude.

Material S7. Ancestral trait estimation for (A) pollination syndromes and (B) altitude.

Material S8. *Vriesea* species distribution across altitude and latitude.

Material S9. Data on pollinator species distribution, habitat preferences, and movement.

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AUTHOR CONTRIBUTIONS

Beatriz Neves, Andrea F. Costa, Fabiano Salgueiro, Christine D. Bacon, and Alexandre Antonelli (study design), Beatriz Neves, Igor M. Kessous, Ricardo L. Moura, and Dayvid R. Couto (sample collection and identification), Beatriz Neves (DNA extraction and preparation), Francisco Prosdocimi, Paola de L. Ferreira, and Beatriz Neves (bioinformatic analyses), Beatriz Neves, Igor M. Kessous, and Paola de L. Ferreira (phylogenetic analyses), Beatriz Neves (remaining analyses), all authors (data interpretation), Beatriz Neves (manuscript writing with contributions from all authors).

DATA AVAILABILITY

Raw reads are available at NCBI under the BioProject number PRJNA918536. The morphological and environmental data used in the analyses are available in the electronic supplement of this paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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