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Chemical Profiles of Floral Structures in *Nicotiana alata* and *N. forgetiana*: Pollen and Corolla Volatiles in Parental Species and Natural Hybrid Corollas

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Abstract

Nicotiana alata (hawkmoth-pollinated) and *N. forgetiana* (hummingbird-pollinated) are two closely related species that were considered reproductively isolated due to their divergent pollination systems. However, the discovery of a natural hybrid zone challenges this view. Floral volatile profiles of these species and their natural hybrids were characterized to identify possible mechanisms and consequences of unexpected gene flow. Organ-specific (corolla, pollen) and diel (day, night) volatile emissions were analyzed using HS-SPME-GC-MS. Corolla scents were dominated by oxygenated monoterpenoids, while pollen blends were rich in microbial-associated compounds, such as acetoin. Hybrid scent profiles more closely match their floral phenotype than their genomic ancestry, with intermediate hybrids emitting blends distinct from those of both parental species. The discovery of a natural hybrid zone, combined with phenotype-specific scent emissions, challenges the view of strict pollinator isolation. Floral scent presents a complex, modular trait that may facilitate hybridization in this system.

Keywords: Chemical Ecology; Floral VOCs; HS-SPME-GC-MS; *Nicotiana*; Pollen Volatiles

Introduction

A wide variety of Volatile Organic Compounds (VOCs) produced by flowers are critical mediators of pollinator attraction and behavior, influencing the reproductive success of flowering plants and evolutionary processes such as speciation and hybridization (Knudsen *et al.*, 1993; Schiestl & Johnson, 2013). VOCs are emitted from multiple flower

structures, including petals, nectaries, and pollen, and are composed mainly of phenylpropanoids, terpenoids, fatty acid derivatives, and nitrogenous compounds, among other classes (Loreto & Schnitzler, 2010; Maffei, 2010; Bertoli *et al.*, 2011; Muhlemann *et al.*, 2014). In pollination biology, the integrated study of visual traits, such as flower color and morphology, and olfactory cues provides a more comprehensive understanding of plant-pollinator attraction strategies (Faegri & van der Pijl, 1979; Fenster *et al.*, 2004; Dobson, 2006).

Advances in headspace sampling and gas chromatography have enabled detailed characterization of floral VOCs under field conditions, revealing not only the blend compositions but also their critical role in pollinator attraction and reproductive isolation (Raguso, 2008; Schiestl, 2015). Floral VOCs may vary widely spatially across populations and geographic ranges, and even among different floral tissues and structures within the same flower and at different times of the day, influencing pollinator visitation and plant reproductive success (Majetic *et al.*, 2007; 2008; 2009). These temporal patterns fine-tune attraction and may reduce interspecific interference when co-flowering species share pollinators. These variations in floral scent are one of the many ways flowers communicate their availability to pollinators.

Multiple floral olfactory cues can serve as advertisements of resources, playing a key role in fine-tuning pollinator accuracy across different scales of interaction (Fenster *et al.*, 2004; Armbruster, 2014). Petal-emitted VOCs generally attract pollinators from a wider range of distances, while VOCs emitted from nectaries and pollen indicate food availability at a closer range or reflect the metabolic activity of pollen-associated microbiota (Dobson & Bergström, 2000; Muhlemann *et al.*, 2014; Schaefer *et al.*, 2004; Schiestl, 2015). If long-range cues mediate initial attraction and short-range cues provide information on reward availability, then fine-scale spatial variation in floral scent can differentially influence pollinator behavior (Raguso & Willis, 2002, 2005; García *et al.*, 2021), supporting the need to analyze volatile emissions separately from different floral structures, such as corolla and pollen.

Olfactory cues represent a critical component of the broader suite of floral signals that mediate plant-pollinator interactions, with their relative importance differing among pollinator groups (Fenster *et al.*, 2004). Hummingbirds, for example, rely mainly on visual cues, such as flower color, morphology, and spatial display, while exhibiting relatively limited dependence on floral scent compared to other pollinators, including

hawkmoths (Fester, 2004; Cronk & Ojeda, 2008; Raguso, 2008). In contrast, many nocturnal pollinators, particularly hawkmoths, depend strongly on specific floral scent blends for host location and discrimination, often responding to precise combinations of volatile organic compounds (Raguso & Willis, 2002; 2005; Policha *et al.*, 2016; 2019). Hawkmoths, the primary pollinators of *Nicotiana alata* Link & Otto, are innately attracted by specific volatile compounds (Haverkamp *et al.*, 2016) and use scent as a long-distance cue, feeding on any visually matching flowers once they enter a scent cloud, regardless of whether those flowers emitted the scent (Raguso & Willis, 2002). Nonetheless, pollinators like hawkmoths and bees can learn to associate odors with rewards, even when visual cues differ or are absent (Daly and Smith, 2000; Wright and Schiestl, 2009; Lawson *et al.*, 2018; Cook *et al.*, 2020). This behavioral flexibility can enhance gene flow and promote the formation of hybrid zones at species contact zones (Rezende *et al.*, 2020; Turchetto *et al.*, 2022).

The genus *Nicotiana* is an excellent model for exploring the interplay among floral scent emissions, pollinator behavior, and hybridization. With over 100 species, *Nicotiana* includes numerous hybrid species, polyploids, and homoploids (Chase *et al.*, 2003; 2021; Clarkson *et al.*, 2004; Knapp, 2020), and exhibits remarkable floral diversity in shape, pollinator rewards, color, and scent emission, attracting a wide range of pollinators (Raguso *et al.*, 2006; Knapp, 2010; Kaczorowski *et al.*, 2012; García *et al.*, 2020; Popova *et al.*, 2020; Couto *et al.*, 2024). Hybridization is well documented in the genus *Nicotiana* due to its role in speciation (Chase *et al.*, 2003; 2021; Clarkson *et al.*, 2004). Because hybridization is a well-known evolutionary driver of plant diversity in many species (Soltis & Soltis, 2000; Schley *et al.*, 2022; Turchetto *et al.*, 2022) and most ancient hybridization events in *Nicotiana* are inferred to have occurred in the Neotropics (Clarkson *et al.*, 2017), we focused on two Neotropical diploid Nicotiana species that naturally form a hybrid zone.

This study examines *Nicotiana alata*, *N. forgetiana* Hemsl., and a natural hybrid population found in a contact zone between the two species in southern Brazil (Figure 1A). The hybrid status of this natural atypical population was initially identified based on its floral morphology and coloration (Teixeira *et al.,* 2022; Couto *et al.*, 2023) and was recently confirmed by genomic data (Couto *et al.*, 2026). The two parental species belong to the *Alatae* section, representing the most recent diploid clade in *Nicotiana* (Clarkson *et al.*, 2017; Knapp, 2020). Both species share n = 9 chromosomes and have

distinct distributions in South American grasslands. *Nicotiana alata* is widespread across southern Brazil, northeastern Argentina, and southern Paraguay, while *N. forgetiana* is restricted to open areas in the southern Brazilian Atlantic Forest. Their ranges overlap in open areas of this region (Teixeira *et al.*, 2022).

Nicotiana alata has strongly scented, night-opening flowers with large, narrow-tubed white corollas, while *N. forgetiana* has smaller magenta flowers with a faint odor that open during the day (Figure 1D) (Kaczorowski *et al.*, 2005; Raguso *et al.*, 2006). Those two species can produce hybrids under controlled laboratory conditions but were considered isolated due to their different pollination systems, hawkmoths and hummingbirds, respectively (Ippolito *et al.*, 2004; Bissell & Diggle, 2008). However, bees have been observed collecting nectar and pollen from *N. forgetiana* and accessing *N. alata* pollen from closed flowers in contact zones (Turchetto *et al.*, 2022). The roles of bees and other animals as secondary pollinators and potential facilitators of hybridization of these species remain to be investigated. The recent finding of sympatric populations of these species with individuals exhibiting atypical phenotypes, ranging from flowers with parental canonical morphology but a mosaic of parental genotype (Figure 1B–C, Couto *et al.*, 2026), to individuals with classic hybrid intermediate features (Figure 1E–F), challenges the previous understanding of complete isolation. In this context, given the unexpected observation of bee visitation in the hybrid zone, we conducted a structure-specific analysis to disentangle the contributions of different floral organs and identify which may be driving pollinator responses.

Previous studies of flower scent across the *Alatae* section focused on corolla emissions and established similarities and differences in the volatile profiles of *N. alata* and *N. forgetiana* (Raguso *et al.*, 2003; 2006). However, the structure-specific emission profiles, corolla, and pollen contributions to the overall bouquet of these species remain uncharacterized (Couto *et al.*, 2024). Because different floral structures can produce distinct volatile blends associated with different ecological functions (*e.g.*, pollinator attraction and reproductive processes), a structure-specific approach is essential to disentangle their respective contributions. Furthermore, the discovery of a natural hybrid population, along with the unexpected observation of bee visitation (Teixeira *et al.*, 2022; Turchetto *et al.*, 2022), highlights the need for deeper investigation into the volatile emission profiles of these species. This study aims to (1) identify structure-specific scent blends at different times of emission in relation to the parental

species' pollination systems; and (2) analyze the emission profiles of hybrid individuals, comparing how their phenotypically defined classes relate to the parental species.

Materials and Methods

Study system and sampling design

The plants used for scent collection were grown in a greenhouse from seeds obtained through open pollination in the field. Details on germination, cultivation, and greenhouse maintenance are described in Couto *et al.* (2023). Seed accessions were obtained from two natural populations of *N. alata*, two populations of *N. forgetiana*, and one natural population of hybrids in a contact zone (Table 1). Populations Forg05 and Ala16 are located in the contact zone, physically close to the hybrid population Hyb01. Populations Forg04 and Ala15 are distant from the contact zone and from each other. However, it is worth noting that previous molecular and morphological studies have shed light on the complex nature of this species system. The taxonomic classification of the magenta-flowered *N. forgetiana* has been questioned based on morphological evidence (Teixeira *et al.*, 2022) and molecular studies (Augsten *et al.*, 2025), suggesting that it should be recognized as two distinct species. One group occurs in canyon regions along the Serra Geral border in Santa Catarina, Brazil, matching the 'Rastroensis' morphotype 1 (Kaczorowski *et al.*, 2005; Teixeira *et al.*, 2022). The other is found in Rio Grande do Sul's interior, in rocky outcrops or forest clearings, exhibiting the morphology previously attributed to *N. forgetiana* (morphotype 2), where it overlaps with *N. alata* (Teixeira *et al.*, 2022). In this research, seeds from *N. forgetiana* morphotype 2 were collected and germinated; herein, they are referred to exclusively as *N. forgetiana*. The hybrid flowers were classified according to their phenotypes: *Nicotiana alata*-like, the flowers that followed canonical *N. alata* appearance; *N. forgetiana*-like, the flowers that followed canonical *N. forgetiana* appearance; and intermediate, the flowers that exhibit classic hybrid intermediate characteristics. The collection of plant material was conducted in accordance with all relevant guidelines and legislation. The necessary permits were obtained in accordance with Brazilian laws for the collection of genetic material for taxonomic and evolutionary research (SISGEN permit number AB0DE7E and SISBIO permit number 68511). One voucher specimen from each population was

collected in the greenhouse and deposited in the herbaria ICN (Universidade Federal do Rio Grande do Sul) and BHCB (Universidade Federal de Minas Gerais) (Table 1).

Volatile collection and consecutive analysis were conducted in January 2023, during the summer in Brazil. Petals and anthers from five *N. alata*, four *N. forgetiana,* and ten hybrid individuals were collected. To ensure corolla tissue samples were pollen-free upon collection, the flowers were marked and emasculated one day before complete anthesis. Corollas were collected whole and placed in a 20-mL headspace vial, which was sealed, yielding one corolla per sample. For pollen samples, open anthers from five flowers from the same individual were collected and immediately placed in a headspace vial, which was then sealed. Collections were conducted at 11:00 h (day) and 02:00 h (night) to coincide with peak pollinator activity (bees and hawkmoths; Rodrigues *et al.*, 2018; Fenske *et al.*, 2018). Ambient controls (empty vials) and bud controls (unopened flower buds) were not included, as the scent profiles of the parental species have already been well characterized (Raguso *et al.*, 2003; 2006). Given our focus on comparative profiling across tissues and hybrid phenotypes, we prioritized sampling of fully opened corollas and anthers.

Identification of VOC profile compounds and descriptive analysis

The sealed vials were kept at room temperature until analysis that same day. Volatile compounds were analyzed using headspace solid-phase microextraction (HS-SPME; Supelco Inc., Sigma-Aldrich) coupled with gas chromatography-mass spectrometry (GC-qMS).

Samples were conditioned for 15 minutes at 30 °C before exposure to a 100-µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber for an additional 30 minutes. The 30 °C conditioning temperature was selected to approximate ambient conditions in the species' natural temperate habitat during the flowering period, minimizing thermal stress and avoiding the induction of artifactual emissions that might occur at higher temperatures. The fiber was then injected into the GC-qMS port at 250 °C for 10 minutes, followed by fiber cleaning for 10 minutes. Analyses were performed using an autosampler (CTC CombiPAL, Zwingen, Switzerland) coupled to a GC-qMS (QP2010, Shimadzu, Kyoto, Japan). All analyses used splitless injections on a polar DB-WAX column (30 m length × 0.25 mm I.D. × 0.25 µm film thickness). The oven temperature program and carrier gas conditions followed Raguso et al. (2003): initial temperature 60 °C held for 3 min, increased at 10 °C/min to 260 °C, and held for 7 min;

helium carrier gas flow rate was 1.0 mL/min. Unlike the solvent extraction method used in that study, we introduced samples via SPME fiber thermal desorption rather than liquid injection, with adsorption parameters optimized for whole corolla and pollen tissues (15 min headspace equilibration followed by 30 min fiber exposure, both at 30 °C). Peaks from total ion chromatograms (TICs) were detected and integrated using Shimadzu's GCMSsolutions software (v. 2.6). Compound putative identification was performed by comparing spectral similarity and retention index (RI) values with the NIST library. Identifications were accepted when both the mass spectral similarity score was ≥75%, and the experimental RI matched the reference within ±10 units. The retention indices were calculated using a C_7-C_{30} alkane series (Supelco, Bellefonte, USA), analyzed under the same conditions as the samples.

Following identification, compound abundances were expressed as relative percentages, calculated from the normalized peak area (Peak $Area_{compound}/Area_{total} \times 100$). This method does not allow absolute quantification of each compound but provides the mean abundance of each compound in a complex blend, which is internally comparable (Valença *et al.*, 2022). The raw compound data are available in Supplementary Material S2.

To visualize blend compositions and their variations across color phases, emission times, and structural origins, class boxplots were generated for each iteration using the ggplot2 package (Wickham, 2016). To quantify the relative mean abundance on time-related variation, diurnal/nocturnal ratios were calculated, and the results were normalized by applying log2-transformation ($Log_2(R)$, R = day abundance/night abundance; Positive log_2 values indicate higher day emissions: +1 = 2-fold, +2 = 4-fold, +3 = 8-fold; negative values indicate higher night emissions: -1 = 2-fold, -2 = 4-fold, -3 = 8-fold).

Multivariate Analysis

To compare volatile profiles among species, hybrid phenotypes, structure emissions (corolla vs. pollen), and diel time emissions, we performed multivariate analysis of relative abundance data using the *vegan* v. 2.7-3 package (Oksanen *et al.*, 2026) in the R environment (R Core Team, 2021). The relative abundance data were transformed using Hellinger transformation (Legendre & Legendre, 2012) to reduce the influence of rare compounds (Kirichenko-Babko *et al.*, 2021). To visualize the overall

variation, we performed non-metric multidimensional scaling (NMDS), an unconstrained method suited for exploratory analysis (Eisen *et al.*, 2022). As a safeguard against data overexploitation, no constrained analysis was performed due to the imbalance in the sample sizes. The NMDS was performed using Bray-Curtis dissimilarity matrices and was run with 999 random starts to ensure a stable solution. To identify the volatile compounds most strongly associated with the observed ordination patterns, we fitted environmental vectors onto the NMDS ordination using the *envfit* function, which projects compounds as vectors whose direction and length represent the strength and significance of their correlation with the sample distribution. All ordinations were plotted with the *ggplot2* v. 4.0.2 (Wickham *et al.*, 2026) package.

To test for significant differences in volatile composition among groups, we performed permutational multivariate analysis of variance (PERMANOVA) using the *adonis2* function in the *vegan* package (Oksanen *et al.*, 2026) with 999 permutations. Due to unbalanced sample sizes across groups, we conducted separate PERMANOVA models for specific biologically relevant comparisons rather than a single global model. The groups tested were pollen and corolla of *Nicotiana alata* vs. *N. forgetiana.* The groups tested for corolla were: *N. alata* vs. N. alata-like hybrids*; N. forgetiana vs.* N. forgetiana-like hybrids*;* intermediate hybrids *vs. N. forgetiana;* and intermediate hybrids vs. *N. alata.* Bray–Curtis dissimilarity matrices were used as the distance metric for all tests.

Results

Thirty-one compounds were putatively identified in corollas and pollen samples of *Nicotiana alata*, *N. forgetiana,* and their natural hybrids (Supplementary Material Table S2). Terpenes account for 13 compounds, including two sesquiterpenes, four oxygenated monoterpenoids, and seven monoterpenes. The remaining 18 compounds include one aromatic ester, three aromatic aldehydes, eight fatty acid derivatives, and six compounds classified as others. Among the six compounds classified as others, three were exclusively found in pollen blends (acetoin, acetic acid, and γ-valerolactone). Oxygenated monoterpenoids were the predominant class in corolla scent (1,8-cineole, α-terpineol, linalool, and terpinen-4-ol) (Figure 2), with 1,8-cineole being ubiquitous and most abundant. In the pollen scent, the dominant compounds were 2,3-butanediol, 4-methyl-1-pentanol, γ-valerolactone, acetic acid, acetoin, and octyl acetate, with acetic

acid being the most abundant compound. The full table of compounds for each individual is available in Supplementary Material S1.

In corolla blends, the blend complexity varied from 28 in *Nicotiana alata*, 18 in *N. forgetiana*, 20 in *Nicotiana alata*-like hybrids, 19 in *N. forgetiana*-like, and 16 in intermediate phenotype hybrids. Compounds exclusive to *N. alata*'s corollas were (*E*)-B-farnesene, benzaldehyde, linalool, and hexanal; and 4-methyl-1-pentanol was exclusively found in intermediate hybrid samples. There were no exclusive compounds in *N. forgetiana*'s corollas. On the other hand, day emissions yielded 25 compounds, whereas night emissions yielded 26.

For pollen blends, richness ranged from 13 in *N. alata* blends to 8 in *N. forgetiana*. Compounds exclusive to *N. alata* pollen blends were 1,8-cineole, 3-octanol, benzyl alcohol, methyl salicylate, α-terpineol, and terpinen-4-ol; 2-methylbutanal was exclusive to *N. forgetiana* pollen.

Time-of-emission ratios show that *Nicotiana alata* corollas nearly doubled scent emission (Log2 mean increase = -0.955) (Table 2), with β-pinene showing the highest mean increase, 4 times the emission (Log2 mean increase = -2.31). *Nicotiana forgetiana* corolla emissions were two times higher than day emissions (Log2 mean increase = 1.04). Pollen emissions in both parental species followed the corolla trend of higher time of emission. In night emissions of *N. alata* pollen, acetoin had the highest increase of all compounds, reaching nearly eight times higher abundance emission (Log2 mean increase = -3.65). Among hybrids, there was no expressive total increase. Still, it all followed the expected trend for their flower phenotypes: *N. alata-like* flowers were nocturnal (Log2 mean increase = -0.485), *N. forgetiana*-like flowers were diurnal (Log2 mean increase = +0.669), and intermediate flowers were nocturnal (Log2 mean increase = -0.037).

NMDS ordinations of overall corolla volatile emission profiles and corolla vs. pollen profiles are presented in Figure 3A and 3B, respectively. The NMDS of volatile corolla compounds (Fig. 3A) revealed no clear grouping among samples, either by identity or by time of emission. The NMDS analysis yielded a stress value of 0.171, indicating a reliable representation of the structure. Environmental vector fitting identified four compounds that were significantly correlated with the ordination ($p < 0.001$): α-pinene and β-pinene, which dominated the positive NMDS1 axis, and 1-hexanol and benzyl alcohol, dominating the negative NMDS1 axis. Consistent with the

ordination, PERMANOVA analysis of overall corolla volatile profiles revealed no significant effect of groups (species and hybrid phenotypes) or time of emission (df = 5, R^2 = 0.303, F = 1.30, p = 0.179), indicating that these factors collectively explained 30.3% of the variation in corolla scent composition. However, this effect was not statistically significant (Fig. 3C). The PERMANOVA comparisons revealed no significant differences between corolla emissions of *N. alata* and *N. forgetiana* (R^2 = 0.307, F = 0.89, p = 0.667; Fig. 3C), though this analysis was limited by small sample size (n = 4). Similarly, no significant differences were detected between *N. alata* and *N. alata*-like hybrids (R^2 = 0.198, F = 0.87, p = 0.605; Fig. 3C), nor between *N. forgetiana* and *N. forgetiana*-like hybrids (R^2 = 0.482, F = 0.93, p = 0.600; Fig. 3C). However, intermediate hybrids differed significantly from both parental species: from *N. alata* (R^2 = 0.332, F = 2.99, p = 0.031) and from *N. forgetiana* (R^2 = 0.411, F = 4.19, p = 0.039) (Fig. 3C).

In contrast, NMDS ordination of both corolla and pollen samples (Figure 3B) showed clear separation between tissue types, with corolla and pollen samples forming distinct clusters. The NMDS stress value was 0.095, indicating an excellent fit to the dissimilarity structure. Environmental vector fitting identified 12 compounds significantly correlated with the ordination (p < 0.001), including: sabinene, β-pinene, 1,8-cineole, limonene, and β-myrcene dominating the NMDS1 positive axis and the corolla clustering; acetic acid and γ-valerolactone dominating the NMDS1 negative axis and the pollen clustering. PERMANOVA testing the effects of structure (corolla vs. pollen), time of emission, and their interaction revealed a highly significant difference in volatile composition (R^2 = 0.578, F = 9.58, p < 0.001), with the model explaining 57.8% of the total variation (Fig. 3C). For pollen samples, PERMANOVA revealed no significant differences between *N. alata* and *N. forgetiana* or between day and night emissions (R^2 = 0.642, F = 0.90, p = 0.667). However, this analysis was constrained by a limited sample size (n = 4) (Fig. 3C).

Discussion

This study aimed to expand knowledge of floral volatile emissions in the *Nicotiana alata-N. forgetiana* species system, focusing on corolla and pollen volatiles separately and including their natural hybrids. The characterization of volatile emissions from whole corollas and anthers using HS-SPME-GC-MS provided organ-resolved, temporally distinct scent profiles for this species system. Despite sharing similar corolla chemical profiles, the parental species' pollen and corolla emissions are fundamentally distinct. The corolla compound profile of canonical *N. alata* and *N. forgetiana* and the parental-like hybrids was similar at both times of emission, but the intermediate phenotypes showed distinctions from both canonical emissions.

Pollen and corolla emissions of *N. alata* and *N. forgetiana* were found to be dominated by monoterpenes and oxygenated monoterpenoids, both known to be highly emitted by Angiosperms (Knudsen *et al.*, 1993; Kesselmeier & Staudt, 1999). The VOC profiles of pollen and corolla of the parental species differed in composition and relative abundance, consistent with previous studies on these structural emissions (Dobson & Bergström, 2000; Piskorski *et al.*, 2011; Caser & Scariot, 2022). Although the canonical species' blends were not significantly different from one another, this result should be interpreted with caution given our current sample size. Compounds belonging to the "cineole cassette" (Raguso *et al.*, 2006) are linked to the conserved terpineol synthase in *Alatae* species (Fähnrich *et al.*, 2011). The enzyme was identified in the petal epidermis and pistils of *N. alata* (Fähnrich *et al.*, 2011); its presence in pollen-producing tissues (*e.g.*, anthers) has not been explored. In pollen blends, compounds such as 1,8-cineole and α-terpineol may originate from the adsorption of corolla volatiles onto the pollen kit rather than from biosynthesis within anther tissue (Dobson & Bergström, 2000).

Compounds commonly associated with microbial metabolism, such as acetoin and 2,3-butanediol, were detected in *Nicotiana* pollen; however, these volatiles are also reported in plant–microbe interaction contexts, and their occurrence in plant tissues cannot be ruled out based on current evidence (Ryu *et al.*, 2003; Sharifi & Ryu, 2018; Farré-Armengol *et al.*, 2017). Similar microbial-associated volatiles, particularly 1-octen-3-ol and other eight-carbon compounds, have been documented in the floral scents of *Yucca brevifolia* Engelm. (Joshua tree) and *Dracula* Luer orchids, where they are thought to play roles in attracting specialized pollinators (Svensson *et al.*, 2016; Policha *et al.*, 2016). In *Dracula* orchids, these compounds are part of the fungal mimicry

to attract mushroom flies (Policha *et al.,* 2019), while in *Yucca* they occur in a plant with specialized pollination (a mutualistic relationship with yucca moths; Smith *et al.*, 2008), but their actual functional role remains to be tested. In contrast, *Nicotiana alata* and *N. forgetiana* are pollinated by hawkmoths and hummingbirds, respectively (Ippolito *et al.*, 2004), with sporadic visits by honeybees also visiting flowers in contact zones (Turchetto *et al.,* 2022). The presence of microbial-associated volatiles in pollen of these *Nicotiana* species is, therefore, unlikely to reflect a highly specialized pollinator strategy. Instead, it might suggest the presence of an active microbiota.

Pollen grains' morphological structure and nutrient-rich composition qualify them as suitable colonization surfaces (Nicolson & Human, 2013; Blackmore *et al.*, 2013), with different plant species composed of distinctive bacterial and fungal microbiomes, and congeneric species having more similar microbiota (Manirajan *et al.*, 2018). Acetoin (2-hydroxybutanone) and 2,3-butanediol are adjacent bacterial and fungal metabolites in the fermentation metabolic pathway (Xiao & Lu, 2014) and are known VOCs produced by rhizobacterium *Bacillus* sp., which can prime plant innate systemic immune response against pathogens in *N. benthamiana* Domin. and other species (Wu *et al.*, 2018; Cofer *et al.*, 2018). Cardinale & Schnell (2024) highlighted the growing body of literature on the seed microbiome, focusing on mutualistic bacteria and fungi, and reiterated the importance of this microbiota and its yet-unknown origin. Rodríguez *et al.* (2018) suggest that pollen grains may serve as a potential source of the seed microbiome, forming a pollen-microbe-seed system for rhizosphere and phyllosphere colonization in new habitats. The identification of fermentation pathway substrates and products in pollen blends on *N. alata* and *N. forgetiana* is an important addition to pollen-microbe-seed system research.

The volatile profiles of the natural hybrids reveal a complex pattern of inheritance that reflects their underlying genomic mosaicism. Importantly, the present hybrid classifications (*N. alata*-like, *N. forgetiana*-like, and intermediate) were based solely on floral phenotype. Genomic data from the maternal plants of these same individuals (Couto *et al.*, 2026) reveal that phenotype is a poor predictor of overall genotype in this system, which is expected for hybrid zones (e.g., Giudicelli *et al*., 2024). For instance, individuals classified as *N. alata*-like in this study (17.1 and 17.3) were offspring of a maternal plant that was phenotypically *N. forgetiana*-like (Hyb17) but had a higher genomic contribution from *N. alata*. This decoupling of morphology from

genome highlights the complex genetic architecture of these traits, and the hybrid's corolla profile mirrors this pattern. The hybrids with parental morphologies' emissions are indistinguishable from the canonical species' emissions, while intermediate hybrids' emissions are distinct from both parents' blends. These results help reconcile the apparent dichotomy between phylogenetic constraints and pollinator-mediated selection on flower emissions (Delle-Vedove *et al.*, 2017). Phylogenetic relationships primarily constrain blend composition, as evidenced by the shared cineole cassette in section *Alatae* (Raguso *et al.*, 2003; 2006). In contrast, pollinator selection strongly shapes emission timing, as shown by distinct diel rhythms that are conserved even in hybrids and depend on their floral phenotype. The hybrid profiles are particularly revealing, demonstrating that the genetic modules controlling blend composition can be inherited independently from those regulating emission timing, leading to mosaic phenotypes. This modularity may be a key genetic mechanism facilitating the evolution of novel scent profiles within the bounds of a conserved phylogenetic background.

The scent profiles of hybrids have important implications for pollinator behavior and, consequently, for gene flow between species. Previous studies suggested that, even though *N. alata* and *N. forgetiana* do not have strong post-zygotic barriers, they are effectively isolated due to their divergent pollination syndromes. However, in sympatry with artificial hybrids, their pollinators begin to visit both parental species (Ippolito *et al.*, 2004). Our findings provide a chemical basis for this behavioral shift, with intermediate hybrids possibly serving as chemical bridges through their distinct scent profiles. Compounding this effect, all species and hybrid classes in this system possess UV-absorbing floral pigments, rendering them visually similar to pollinators with UV-sensitive vision (Couto *et al.*, 2023). In the absence of strong visual differentiation, scent becomes the primary cue for pollinator decision-making. Once attracted by the "scent cloud" of one flower type, whether from a parent or a hybrid, hawkmoths may subsequently visit other flowers in the area that share similar UV-absorbent patterns, even if those flowers differ in color or scent intensity (Raguso & Willis, 2002). This breakdown of pollinator constancy, facilitated by hybrid scent profiles and visual uniformity, likely contributes to the extensive introgression observed in contact zones (Couto *et al.*, 2026). This study provides the first comprehensive, organ-resolved, and temporally explicit volatile profiles for *Nicotiana alata*, *N. forgetiana*, and their natural hybrids, revealing the multifaceted and dynamic nature of their floral emissions. The

results confirmed that scent composition is largely phylogenetically conserved yet also demonstrate that emission timing is finely tuned by pollinator selection, a duality clearly illustrated by the mosaic scent profiles of the hybrids. Unlike other systems in which hybrids exhibit strictly parental or intermediate emissions (*e.g.*, García *et al.*, 2023; Bischoff *et al.*, 2013), the complex inheritance patterns observed highlight the value of this study system. Future research should focus on the chemical ecology of pollination in these southern Brazilian grasslands to understand the impact of secondary contact on species boundaries and the evolution of pollinator-mediated signals.

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Author's Contributions

MAMSC and CT conceptualized the article, and MAMSC ran the analyses and wrote the initial draft. RAJ and ASP conducted the extraction and analysis. All authors contributed to writing and reviewing the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability Statement

The data underlying this article are available in the article and on its online supplementary material.

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Supplementary Material

The following online material is available for this article:

Supplementary Material S1: Compounds identification per individual.

Supplementary Material S2: Relative peak area of the volatiles emitted by corolla and pollen of *Nicotiana* species and hybrids.

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Table and Figure Captions

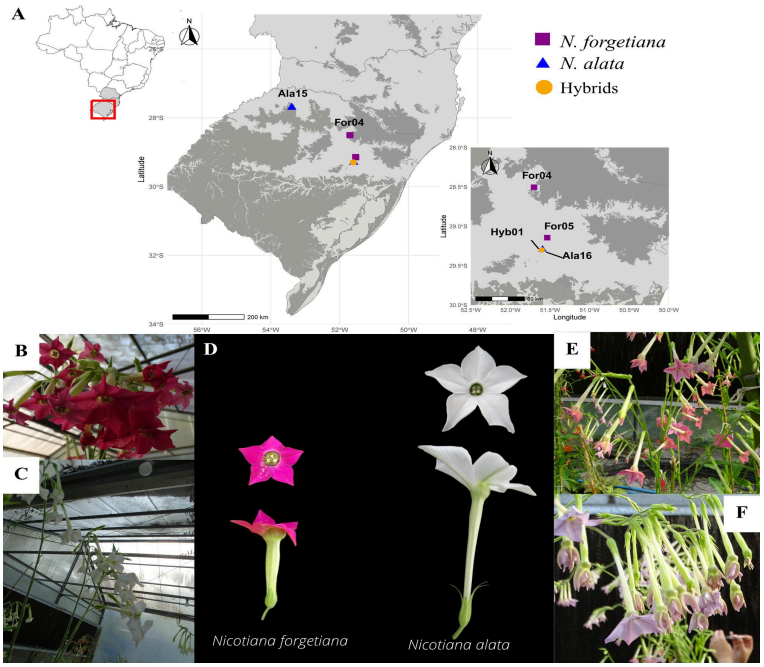

Figure 1: The geographical location of *Nicotiana* species populations, hybrid populations, and plant phenotypes. A: Geographical map of populations; B: *N. forgetiana*-like hybrid; C:

N. alata-like hybrids; D: canonical allopatric *N. alata* and *N. forgetiana* morphology; E and F: intermediate phenotype hybrids.

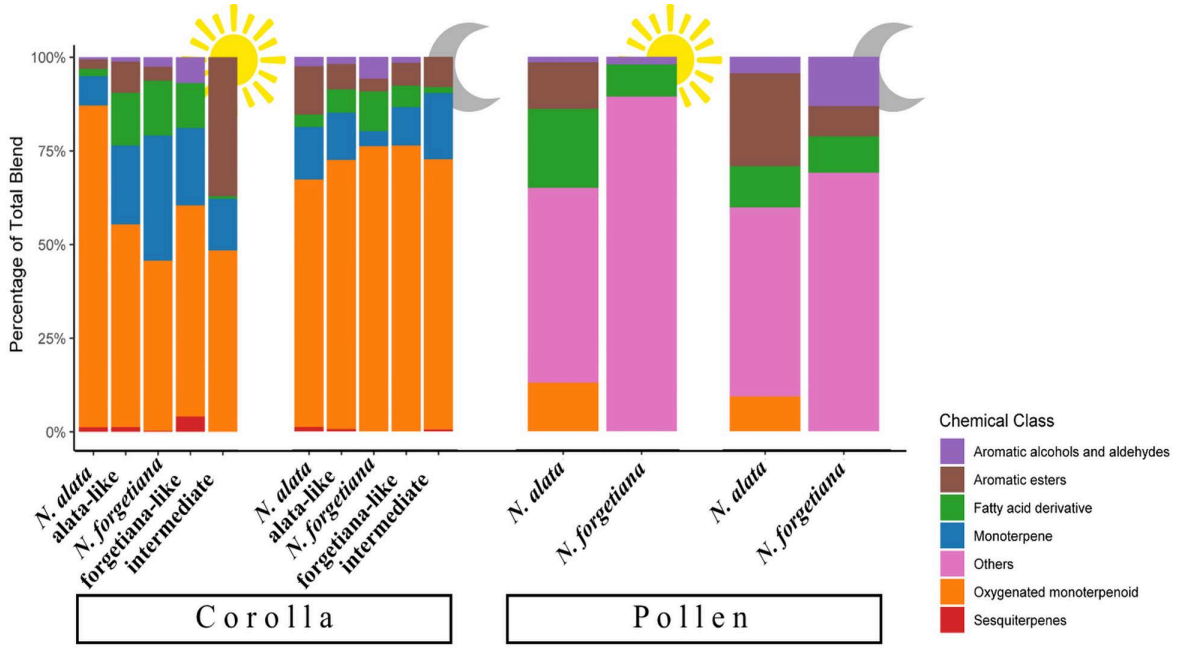

Figure 2: Diversity of floral VOCs in *Nicotiana* species and different hybrid phenotypes at various times of emissions and structures.

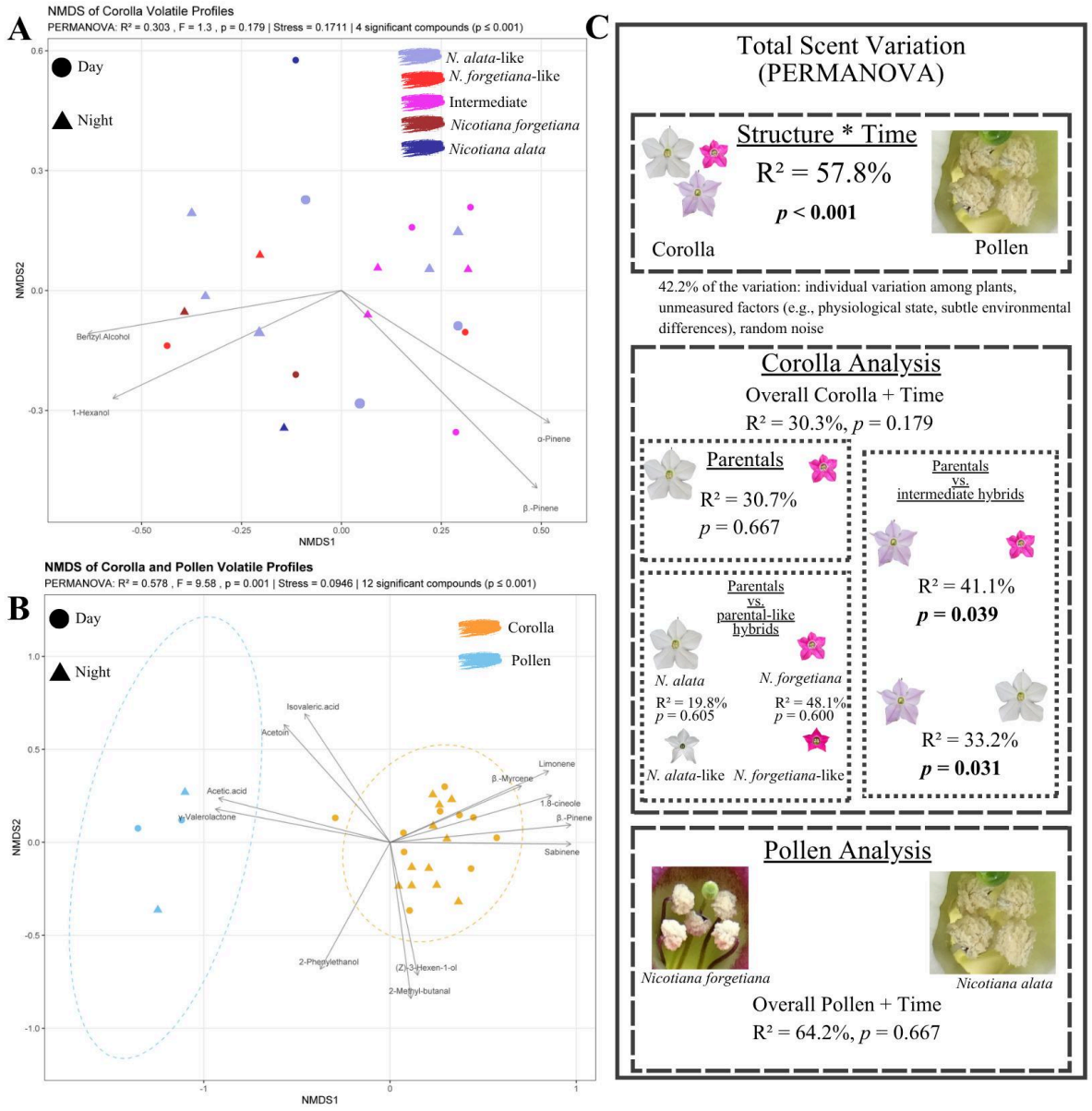

Figure 3: Ordination analysis and PERMANOVA results of VOC of *Nicotiana* species and different hybrid phenotypes at various times of emissions and structures. A: NMDS of corolla volatile profiles, circles: day emissions; triangles: night emissions B: NMDS of corolla and pollen volatiles. C: Diagram of total scent variation PERMANOVA results. Legend: *N. forgetiana* (dark red/brown), *N. forgetiana*-like (light red), Intermediate (magenta), *N. alata*-like (light blue), *N. alata* (dark blue). , day emissions (circles); night emissions (triangles); pollen (light blue); corolla (orange).

Table 1. Sampled data of *Nicotiana* species used in this study. The seeds were collected in the allopatric population of *Nicotiana alata* (Ala) and *N. forgetiana* (Forg), and one hybrid (Hyb) population (Couto et al., 2023); Sampling size (*n*) = one flower/corolla and five flowers/pollen, all from the same single individual.

POP/ID	Geographic coordinate	Voucher	Sampling (*n*)	ID offspring	Class phenotype	Corolla		Pollen	
						Day	Night	Day	Night
Nicotiana alata									
Ala15/302	29°02'30.3"S	BHCB 202381	2	ala302.1	ALAlike	X	X		
	51°33' 56.6"W		5	ala302.2					X
Ala16/318	29°17'38.3"S	BHCB 202376	11	ala318.1	ALAlike	X		X	X
	51°35'39.1"W								
Ala16/328	29°17'38.3"S		7	Ala328.3	ALAlike	X	X		X
	51°35'39.1"W			Ala328.5	ALAlike	X	X		X
Nicotiana forgetiana									
Forg04/72	28°30'19.0"S	BHCB 202363	12	forg72.7	FORGlike	X	X	X	X
	51°42'02.7"W								
Forg05/109	29°08'42.0"S	BHCB 202380	12	forg109.3	FORGlike	X	X	X	X
	51°32'01.7"W								
Forg05/121			12	forg121.1	FORGlike	X	X	X	X
			7	forg121.2	FORGlike	X	X	X	
Hyb01/04	29°18'02.9"S	ICN 201537	2	04.2	ALAlike	X	X		
	51°36'01.6"W	ICN 201538							
		ICN 201539	1	04.3	ALAlike		X		
Hyb01/09		ICN 201540	1	09.2	ALAlike		X		
Hyb01/11			2	11.3	INTERlike	X	X		
			2	11.5	INTERlike	X	X		
			2	11.8	INTERlike	X	X		
Hyb01/17			2	17.1	ALAlike	X	X		
			2	17.3	ALAlike	X	X		
Hyb01/31			1	31.6	FORGlike	X			
Hyb01/35			2	35.3	FORGlike	X	X		

Table 2: Diurnal and nocturnal VOC emissions in *Nicotiana* species and hybrids in different structures.

Species. structure	Compound	Time of emission (% relative abundance)	Fold variation (log2)	Predominance
N. alata				
Corolla				
	β-Pinene	DAY: 0.32 NIGHT:1.60	-2.31	Night
	(*Z*)-3-Hexen-1-ol	DAY:0.58 NIGHT:2.1	-1.85	Night
	α-Terpineol	DAY:0.18 NIGHT:0.56	-1.65	Night
	Sabinene	DAY:2.01 NIGHT:6.29	-1.65	Night
	Isoamyl alcohol	DAY:4.42 NIGHT:1.49	+1.57	Day
	Methyl salicylate	DAY:1.12 NIGHT:3.09	-1.47	Night
	β.-Myrcene	DAY:2.47 NIGHT:5.09	-1.04	Night
		Total increase	**-0.95**	**Night**
Pollen				
	Acetoin	DAY: 1.38 NIGHT:17.4	-3.65	Night
	2-Phenylethanol	DAY: 0.94 NIGHT:2.96	-1.65	Night
	Methyl salicylate	DAY: 2.54 NIGHT:7.32	-1.53	Night
	Isovaleric acid	DAY: 21.2 NIGHT:10.5	+1.01	Day
		Total increase	**-0.48**	**Night**
N. forgetiana				
Corolla				
	Limonene	DAY: 12.6 NIGHT:0.58	+4.50	Day
	α-Ocimene	DAY: 17.3 NIGHT:1.91	+3.18	Day
	Isovaleraldehyde	DAY: 1.96 NIGHT:0.51	+1.95	Day
	Isoamyl alcohol	DAY: 17.6 NIGHT:5.06	+1.81	Day
	β -Pinene	DAY: 1.81 NIGHT:0.58	+1.63	Day

	β.-Myrcene	DAY: 4.99 NIGHT:1.84	+1.43	Day
	1.8-cineole	DAY: 29.8 NIGHT:63.8	-1.10	Night
		Total increase	**+1.04**	**Day**
Pollen				
	2-Phenylethanol	DAY: 1.39 NIGHT:12.3	-3.14	Night
	Isovaleraldehyde	DAY: 0.13 NIGHT:0.74	-2.49	Night
	Isoamyl alcohol	DAY: 12.7 NIGHT:25.5	-1.01	Night
		Total increase	**+0.05**	**Day**
Hybrids				
N. alata-like				
	Isovaleraldehyde	DAY: 0.13 NIGHT:1.4	-3.39	Night
	α-Ocimene	DAY: 6.75 NIGHT:0.76	+3.16	Day
	α-Thujene	DAY: 0.72 NIGHT:0.17	+2.04	Day
		Total increase	**-0.48**	**Night**
N. forgetiana-like				
	Limonene	DAY: 6.15 NIGHT:2.43	+1.34	Day
	Benzyl alcohol	DAY: 2.63 NIGHT:1.07	+1.3	Day
		Total increase	**+0.67**	**Day**
Intermediate				
	Methyl salicylate	DAY: 10.9 NIGHT:1.82	+2.58	Day
	α-Ocimene	DAY: 0.51 NIGHT:1.71	-1.73	Night
		Total increase	**-0.04**	**Night**

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