UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-graduação em Biologia Vegetal

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OS TABACOS BRASILEIROS (*NICOTIANA* SECT. *ALATAE*, SOLANACEAE) REVISITADOS À LUZ DE ESTUDOS FILOGENÉTICOS MOLECULARES

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Gastei uma hora pensando em um
verso que a pena não quer escrever.
No entanto ele está cá dentro inquieto, vivo.
Ele está cá dentro e não quer sair.
Mas a poesia deste momento inunda minha vida inteira.

Carlos Drummond Andrade

Catar feijão se limita com escrever:
Jogam-se os grãos na água do alguidar
E as palavras na folha de papel;
e depois, joga-se fora o que boiar.

João Cabral de Melo Neto

RESUMO GERAL

Nicotiana L. é um gênero de Solanaceae de relevância global que conta com quase 100 espécies distribuídas entre Américas, África (uma espécie), Austrália e ilhas oceânicas, com origem e diversificação na América do Sul, mais especialmente relacionada aos Andes. O gênero é dividido em 13 seções, e apenas uma delas, *N.* sect. *Alatae* Goodsp., se diversificou no leste do continente sul-americano, região do sul e sudeste do Brasil. Esta seção, composta por dez espécies, é revisitada neste estudo realizado à luz da taxonomia integrativa, utilizando primariamente estudos filogenéticos moleculares e modelagem de nicho ecológico. O primeiro capítulo trata da descrição de *N. gandarela* Augsten & Stehmann, uma espécie recém descoberta e conhecida de uma única população ocorrente na Serra do Gandarela em Minas Gerais, habitando ambientes sombreados junto a afloramento rochoso no interior da floresta. Realizamos, além da descrição, a filogenia molecular incluindo sete espécies de N. sect. Alatae e ao menos um representante de cada uma das treze demais seções, para verificar a qual seção N. gandarela pertence. Os dados moleculares foram obtidos de sequenciamento Sanger, de regiões de DNA nuclear (ITS) e de cloroplasto (gene ndhf e os espaçadores trnL-trnF, trnS-trnG). Os resultados evidenciaram que N. gandarela é a espécie grupo irmão das demais espécies da seção Alatae, e está criticamente ameaçada de extinção. No segundo capítulo realizamos investigamos *N. forgetiana* Hemsl. s.l., espécie descrita em 1904, e cujo nome é utilizado para todas as espécies de *Nicotiana* de flor magenta ocorrendo na Serra Geral, nos estados do Rio Grande do Sul e Santa Catarina. Trabalhos de ecologia anteriores sugeriam que as populações destas plantas que ocorriam nos cânions dos Aparados da Serra constituíam-se em verdade de uma espécie distinta daquela que ocorre no interior do estado do Rio Grande do Sul, na região ecotonal entre a floresta mista e os Campos de Cima da Serra. Neste estudo, utilizamos as regiões intergênicas de cloroplasto (*trnS-trnG*, *rps12-rpl20* and *rpl32-trnL*), bem como modelagem de nicho ecológico para o presente e para o passado. Nossos achados indicaram a existência de dois grupos monofiléticos com suporte robusto, onde os indivíduos em cada grupo não compartilham haplótipos e não há sobreposição de nicho ecológico entre os grupos. Em concordância com estudos morfométricos anteriores, confirmamos que as populações localizadas nas bordas dos planaltos de Santa Catarina e do nordeste do Rio Grande do Sul representam uma espécie distinta. Os dois estudos realizados oferecem evidências de que as regiões sul e sudeste do Brasil foram palco de importantes processos de diversificação da *N.* sect. *Alatae* e destacam ser o grupo um excelente modelo para estudos evolutivos.

Palavras-chaves: complexos de espécie, filogenia molecular, modelagem de nicho, Serra Geral, tabaco

OVERALL ABSTRACT

Nicotiana L. is a genus of Solanaceae of global relevance that has almost 100 species distributed among the Americas, Africa (one species), Australia and oceanic islands, with origin of diversification in South America, more especially related to the Andes. The genus is divided into 13 sections, and only one of them, *N.* sect. *Alatae* Goodsp., has diversified in the east of the South American continent, in the south and southeast of Brazil. This section, composed of ten species, is revisited in this study carried out in the light of integrative taxonomy, using primarily molecular phylogenetic studies and ecological niche modeling. The first chapter deals with the description of *N. gandarela* Augsten & Stehmann, a recently discovered and known species from a single population occurring in Serra do Gandarela in Minas Gerais, inhabiting shaded environments next to rock outcrops inside the forest. We performed, in addition to the description, the molecular phylogeny including several representatives of *N.* sect. *Alatae* and at least one representative from each of the other sections to verify the phylogenetic position of the species in the genus. Molecular data were obtained from Sanger sequencing, from nuclear (ITS) and chloroplast DNA regions (*ndhf* gene and *trnL*-*trnF*, *trnS*-*trnG* spacers). The results showed that it is the sister group of the other species in the section and that it is critically endangered. In the second chapter we investigated *N. forgetiana l.s.* species described in 1904, and whose name is used for all species of pink-flowered Nicotiana occurring in Serra Geral, in the states of Rio Grande do Sul and Santa Catarina. Previous ecology works suggested that the populations of these plants that occurred in the Aparados da Serra canyons actually constituted a different species from the one that occurs in the interior of the state of Rio Grande do Sul, in the ecotonal region between the mixed forest and the grasslands. from the top of the mountain. In this study, we used the chloroplast intergenic regions (*trnS*-*trnG*, *rps12*-*rpl20* and *rpl32*-*trnL*) as well as ecological niche modeling for the present and the past. Our findings indicated the existence of two monophyletic groups with robust support divided according to their morphological classification, where individuals in each group do not share haplotypes and there is no ecological niche overlap between groups. In agreement with previous morphometric studies, we confirmed that populations located on the edges of the plateaus of Santa Catarina and northeastern Rio Grande do Sul represent a distinct species. The two studies carried out provide evidence that the southern and southeastern regions of Brazil were the scene of important *N.* sect *Alatae* diversification processes and highlight that the group is an excellent model for evolutionary studies.

Keywords: species complexes, molecular phylogeny, niche modeling, Serra Geral, tobacco

Sumário

INTRODUÇÃO GERAL

O Brasil possui mais de 46 mil espécies de plantas, algas e fungos, sendo um dos países com maior biodiversidade do planeta (BFG, 2018). Conhecer e catalogar sua flora é um trabalho contínuo: no Brasil uma espécie nova é descrita a cada dois dias (Sobral & Stehmann, 2009). A formação de botânicos é um passo fundamental, pois são essas pessoas com treinamento que são responsáveis pela maioria das coletas em herbários (Ahrends *et al.*, 2011) e fornecem os dados para a base do conhecimento sobre a flora nativa. Se o objetivo é frear a contínua perda de biodiversidade (CBD, 2010) conhecer e catalogar a flora é uma meta fundamental. O trabalho colaborativo de botânicos no Brasil foi responsável por organizar e sistematizar a flora, alcançando a primeira das metas da Estratégia Global para a Conservação de Plantas (2011-2020), que era "produzir uma flora online de todas as plantas conhecidas" (BFG, 2018).

Neste contexto, na busca por um grupo de plantas que necessitasse de revisão taxonômica que pudesse servir de modelo para estudos evolutivos, viemos ao encontro do grupo nativo de espécies do gênero *Nicotiana* (Solanaceae), as 'Nicotianas Brasileiras'. Todas essas espécies nativas, com diversificação no sul e sudeste do Brasil pertencem à seção chamada *Nicotiana* sect. *Alatae*.

Nicotiana L. é popularmente conhecido por ser o gênero do tabaco. Sua diversidade contudo, vai muito além de *Nicotiana tabacum* L. e *N. rustica* L., as espécies utilizadas como matéria prima na confecção de cigarros e charutos. O gênero possui aspectos evolutivos interessantes, com quase metade das espécies originárias de processo de hibridização (Chase, 2003; Knapp, 2020). Muitas espécies são alopoliploides, e há uma grande variação no número cromossômico entre as espécies e grupos de espécies. *Nicotiana* fez parte de estudos genéticos desde o início do século passado (East, 1916; Anderson & De Winton, 1931), e inclui a primeira descrição do genoma do cloroplasto, referência ainda usada por praticamente todos, quando é preciso localizar genes, ou se referir à estrutura do cloroplasto (Shinozaki *et al.*, 1986). *Nicotiana* também é o gênero de *N. benthamiana* Domin., uma planta que devido a uma mutação específica no gene da RNA polimerase, apresenta-se susceptível a infecção por diversos vírus. Por este motivo, esta linhagem, conhecida como LAB, tem sido utilizada como uma "biofábrica" para produção de compostos farmacêuticos, incluindo até mesmo vacina contra a COVID-19 (Chase *et al.*, 2021). Em relação ao aspecto sistemático, o gênero continua sendo destaque, sendo um dos primeiros a ter diferentes filogenias moleculares publicadas, com diferentes marcadores testados (Aoki & Ito, 2000; Chase, 2003; Clarkson *et al.*, 2004), que levaram a revisão da classificação infragenérica do grupo, com revisão das seções (Knapp, Chase, & Clarkson, 2004). Além disso, continuamente novas espécies têm sido descritas, em especial para a seção Australiana (*N.* sect. *Suaveolentes* Goodsp.), desde a última monografia sobre o gênero (Goodspeed, 1954; Chase *et al.*, 2018; Santilli *et al.*, 2021).

Várias seções de *Nicotiana* possuem sua diversificação relacionadas aos Andes, que também é a origem do gênero. *Nicotiana* sect. *Alatae,* por sua vez, apresenta a maioria das espécies com distribuição restrita ao sul e sudeste do Brasil, com espécies endêmicas, e uma concentração da diversidade na região sul. Suas plantas são ervas anuais, algumas formando rosetas, com folhas com limbo decurrente, muitas vezes apresentando um caule alado, característica que dá o nome ao grupo.

O objetivo geral desta tese foi aplicar a abordagem da taxonomia integrativa (Dayrat, 2005) usando como modelo *N.* sect. *Alatae*, na orientação da tomada de decisões taxonômicas, seja no reconhecimento de novas espécies quanto na elucidação da resolução de complexos de espécies. Taxonomia integrativa é a ciência por trás da delimitação das unidades evolutivas, as espécies (De Queiroz, 2007), através de perspectivas múltiplas e complementares. Pode-se dizer que a taxonomia integrativa possui um preceito fundamental, prioriza a delimitação de espécies ao invés da criação de novos nomes (Dayrat, 2005). *N.* sect. *Alatae* possuía algumas lacunas de conhecimento taxonômico que precisavam ser revistos, e por isso dois trabalhos principais foram realizados.

No primeiro capítulo, descrevemos *Nicotiana gandarela* Augsten & Stehmann, uma planta que havia sido recentemente descoberta e necessitava de uma descrição formal. Contudo, a fim de cumprir com boas práticas para descrição de novos táxons, buscou-se realizar não somente a descrição, mas também estudos filogenéticos moleculares para verificar o pertencimento da espécie à seção. Isso foi feito porque, apesar de várias outras semelhanças morfológicas (hábito herbáceo, folhas em rosetas, e morfologia típica zigomórfica lembrando outras espécies da seção, como *N. bonariensis* Lehm.), a espécie não possuía a dilatação antes da abertura do tubo, além de ocupar um habitat não convencional em relação às outras espécies.

No segundo capítulo, focado em grupo de plantas que ocorrem na região do sul do país, fomos buscar evidências moleculares e biogeográficas para a tomada de decisão acerca da identidade das Nicotianas rosadas que ocorrem nos paredões dos cânions da Serra Geral, sugerida por outros autores como uma espécie distinta ainda não descrita.

O déficit Lineano, ou a lacuna de conhecimento de quantas espécies realmente existem, é ainda mais pronunciado quando tratamos de complexos de espécies e espécies crípticas (Walters *et al.*, 2021). A taxonomia integrativa busca confrontar dados por diferentes abordagens, afim de reafirmar as hipóteses, no caso, as espécies, e tem sido apontada como um caminho, para superar o impedimento taxonômico (Dayrat, 2005; Engel *et al.*, 2021), sendo uma excelente prática para grupos onde a delimitação entre as espécies não é tão claro, como os complexos de espécie. Sendo assim, espera-se diminuir o déficit

Lineano do grupo no Brasil, além de incentivar o uso de abordagens múltiplas e complementares na tomada de decisão taxonômica, através da chamada taxonomia integrativa.

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CAPÍTULO 1. “Nicotiana gandarela (Solanaceae), a new species of ‘tobacco’ highly endangered from the Quadrilátero Ferrífero in Brazil”

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Nicotiana gandarela (Solanaceae), a new species of 'tobacco' highly endangered from the Quadrilátero Ferrífero in Brazil

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Abstract

Nicotiana gandarela Augsten & Stehmann (Solanaceae), **sp. nov.**, a small 'tobacco' known only from one locality at Serra do Gandarela, in the state of Minas Gerais, Brazil, is described and illustrated. It is morphologically characterized by its rosulate basal leaves, red corolla with a short tube not inflated at the apex, and the peculiar habitat, a shaded site under a rocky outcrop ledge along a forested stream. Phylogenetic analyses based on a combined dataset of nuclear (ITS) and plastid (*ndhF*, *trnLF*, and *trnSG*) DNA sequences revealed that the species belongs to the *Nicotiana* sect. *Alatae* and is sister to the clade with the remaining species in the section. A key for the identification of Brazilian species of the section is given. The unusual habitat, the small population size, and the intense pressure of mining activities in the surroundings made the species assessed as Critically Endangered (CR), needing conservation efforts to avoid its extinction.

Keywords

Endemism, IUCN, Minas Gerais, mining, molecular phylogeny

Introduction

Nicotiana L. is a Solanaceae genus known mostly because of tobacco (*N. tabacum* L.), a crop cultivated worldwide, but its diversity goes further. It is the fifth-largest genus in the family, with 82 species (Knapp 2020). *Nicotiana* occurs in South America and Australia, with one species from Africa (Namibia). The Andean region is recognized as the center of diversity of the group in the Americas, as for many other clades in Solanaceae (Goodspeed 1954; Dupin et al. 2017). Molecular phylogenies are available for the genus (Aoki and Ito 2000; Chase et al. 2003; Clarkson et al. 2004; Clarkson et al. 2017), and sectional revision is provided based on phylogenetic analyses, indicating 13 sections (Knapp et al. 2004).

In Brazil, the known native species belong to the *Nicotiana* sect. *Alatae* Goodsp. (hereafter Alatae). This section is morphologically characterized by herbaceous habit, rosulate and sessile leaves, usually viscid pubescent, few leaves on upper stems, and an abrupt dilatation at the throat of the corolla tube (Knapp et al. 2004). The section comprises two cytogenetic groups: one displays chromosome number 2n = 20 and includes two species, *N. longiflora* Cav. and *N. plumbaginifolia* Viv., distributed from Argentina to the USA; the other presents chromosome number 2n = 18 and includes *N. alata* Link & Otto, *N. bonariensis* Lehm., *N. forgetiana* Hemsl., *N. langsdorffii* Weinm., *N. mutabilis* Stehmann & Semir and *N. azambujae* L. B. Sm. & Downs, occurring in Brazil, Argentina, Uruguay, and Paraguay (Goodspeed 1954; Clarkson et al. 2004; Knapp et al. 2004). The internal phylogenetic relationships of the section are still not well resolved, with the two groups recovered as sisters (Clarkson et al. 2004) or with the 2n = 18 group as paraphyletic (Clarkson et al. 2017). According to the plastid or nuclear markers used for the reconstruction, section diversification is proposed between 6.2 Ma and 7.7 Ma, respectively (Clarkson et al. 2017).

Nicotiana can be considered a taxonomically well-studied genus for the Brazilian flora (Vignoli-Silva and Mentz 2005; Vignoli-Silva and Stehmann 2020), in which six native, *N. alata*, *N. bonariensis*, *N. langsdorffii*, *N. forgetiana*, *N. mutabilis*, and *N. azambujae*, and two naturalized species, *N. tabacum*, and *N. glauca* Graham, are recognized. Morphometric analyses based on the corolla size and shape of *N. forgetiana* throughout its geographical distribution showed that the species seems to include two distinct taxonomic groups, one not yet formally described (Teixeira et al. 2021). Among the Brazilian species, only *N. mutabilis* is considered endangered (Vulnerable) because of its small geographic distribution and just a few known populations (Stehmann et al. 2002; CNCFlora [http://cncflora.jbrj.gov.br/portal/pt-br/profile/Nicotiana%20mutabilis]). *Nicotiana azambujae* needs further investigation, as it is only known from the type collection.

Within Brazil, the Southern region holds the largest number of *Nicotiana* species, which inhabit ecotonal environments between the grasslands and the *Araucaria* forest (Mixed Ombrophilous Forest) in the Atlantic Forest domain (Teixeira et al. 1986; Overbeck et al. 2007). This is an important biogeographic area for several taxa that have their evolutionary history related to this vegetation mosaic (Iganci et al. 2011;

Fregonezi et al. 2013). Species richness decreases towards the north, with *N. bonariensis* being the species with the northern-most distribution, reaching the grasslands at the high altitude regions of the Mantiqueira Range (Minas Gerais and Rio de Janeiro states) and the Espinhaço Range (Minas Gerais and Bahia states).

During floristic studies in the Quadrilátero Ferrífero, a mountainous area with large mineral reserves in Minas Gerais, with significant biological importance and high anthropic pressures (Drummond et al. 2005; Jacobi et al. 2011), samples of a small *Nicotiana* specimen with red flowers were collected. As the phenotype matched with none of the known *Nicotiana* species from Brazil, we assumed it could be a new species. In this work, we test this hypothesis using morphological and molecular data.

Methods

Taxonomy

Plant samples were collected during field expeditions in April 2018, and voucher specimens were deposited in the BHCB herbarium at the Universidade Federal de Minas Gerais, whereas flowers were preserved in Ethanol 50%. We took measures of vegetative parts from herbarium samples and of entire flowers from fixed material. Descriptive terminology was based on Radford et al. (1976). To compare the morphological characters with congeneric species and build the key, we revised the main taxonomic literature (Goodspeed 1954; Vignoli-Silva and Mentz 2005; Cocucci 2013; Vignoli-Silva and Stehmann 2020), including the protologues, as well as the materials from the following Brazilian herbaria: BHCB, HUEFS, ICN, MBM, OUPR, PAMG, RB, R, SP, SPF, and UEC. We also examined high-resolution images of the types available at Global Plants (https://plants.jstor.org).

Sampling for phylogenetic analyses and molecular methods

We included DNA sequences from 56 *Nicotiana* species, mostly generated in previous studies (Chase et al. 2003; Clarkson et al. 2004) and obtained from GenBank (www.ncbi.nlm.nih.gov/Genbank). We selected species from all described sections (Knapp et al. 2004) and used two *Anthocercis* Labill. as outgroups. We generated DNA sequences for three different individuals of the new species, also for *N. mutabilis*, described in 2002 (Stehmann et al. 2002) and for which there were no DNA sequences available in the databases. For all Genbank accessions used in this work, see Suppl. material 1: Table S1. Fresh, young leaves were collected in the field and stored in silica gel. Total DNA was obtained using a modified version of the 2X-CTAB extraction method (Doyle and Doyle 1987) using 50 mg of tissue mass and washing with 70% ethanol. We used nucleotide sequences from the nuclear marker (ITS - rDNA internal transcribed spacers) and three plastid regions (*trnLF*, *trnSG*, and *ndhF*). The selection of markers was based on previous studies (Chase et al. 2003; Clarkson et al. 2004). Amplifications were performed using the same conditions described in previous works (Chase et al. 2003; Clarkson et al. 2004). Polymerase chain reaction (PCR) products were purified by precipitation with 20% PEG (2.5 M NaCl and 20% polyethylene glycol 8000) and sequenced on demand by a specialized company using the same primers employed for amplification. We obtained bidirectional sequence reads for all the DNA regions, and consensus sequences were obtained using Geneious Prime 2020.0.3 (https://www.geneious.com). Sequences were aligned using CLUSTALW (Larkin et al. 2007) followed by manual adjustments in MEGA 11 (Tamura et al. 2021). Sequences were concatenated with MESQUITE (Maddison and Maddison 2019), and we obtained the best substitution model per DNA region using MRMODELTEST2 (Nylander 2004).

Phylogenetic analyses

We analyzed the data using Bayesian inference (BI) and Maximum Likelihood (ML). Initially, nuclear and plastid DNA were separately analyzed and posteriorly combined. We performed BI analyses using MRBAYES 3.2.7a (Ronquist et al. 2012) as implemented in the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal 2.0 (Miller et al. 2010), treating each DNA region as a separate partition. The "unlink" command was used to estimate model parameters separately for each partition. Each analysis consisted of two independent runs with four chains for 7,500,000 generations, sampling one tree every 1,000 generations and a temperature parameter of 0.2. Convergence between the runs was evaluated using the average standard deviation of split frequencies (< 0.01) and the Potential Scale Reduction Factor – PRSF (= 1.0) and was achieved after 715,000 generations. After discarding the first 2,500 trees (33%) as the burn-in, the remaining trees were used to assess topology and posterior probabilities (PP) in a 50% majority-rule consensus. We considered groups with PP > 0.95 as strongly supported, groups with PP ranging from 0.90–0.95 as moderately supported, and groups with PP < 0.90 as weakly supported. Maximum likelihood (ML) analysis of the concatenated dataset was performed using the online version of RAxML-HPC BlackBox (v.8.2.12) (Stamatakis 2014) through the CIPRES Science Gateway Portal (Miller et al. 2010). Bootstrap values were calculated based on 1000 replicates.

Conservation status

The conservation status was assessed based on the International Union for Conservation of Nature Criteria (IUCN 2019), considering the main threats to the Quadrilátero Ferrífero vegetation (Sonter et al. 2014).

Scanning electron microscopy analysis

Seeds were affixed in aluminum stubs using double-sided carbon tape, metalized with 10 nm gold-palladium (Robards 1978). The observations were performed under a JEOL JSM– 6360LV scanning electron microscope, with 5 kV (Jeol Ltd., Tokyo, Japan).

Results

Alignment characterization

The concatenated matrix consisted of 3,483 aligned characters, of which 317 (9.1%) were potentially informative. The ITS region had, proportionately, the highest number of phylogenetically-informative characters (20%). General features of the datasets and a summary of the models implemented for each partition are presented in Table 1.

Table 1. General features of the markers used in the phylogenetic analyses and models implemented for each.

Regions	Terminals	Characters	Variable uninformative characters	Informative characters (%)	Model
ITS	59	683	89	135 (20%)	GTR+I+G
ndhF	60	1056	49	71 (6.7%)	GTR+G
trnLF	60	991	41	58 (5.8%)	GTR+G
trnSG	60	855	38	62 (7.2%)	GTR+G
All plastid regions	60	2799	125	182 (6.5%)	–
All regions	60	3483	214	317 (9.1%)	–

Phylogenetic relationships

In the Bayesian phylogenetic tree based on ITS, *N. gandarela* appears within the section Alatae, the Alatae clade is moderately supported (PP = 0.91), and *N. gandarela* is sister of the 2n = 20 clade (*N. longiflora* and *N. plumbaginifolia*), being weakly supported (PP = 0.76) (Suppl. material 2: Fig. S1). The concatenated plastid regions recovered a Bayesian phylogenetic tree with *N. gandarela* as the sister species of section Noctiflorae, moderately supported (PP = 0.91), and Alatae appears as sister of section Repandae, weakly supported (PP = 0.84) (Suppl. material 3: Fig. S2).

Due to the combined dataset produced higher-supported trees in both ML and BI methods than using nuclear and plastid regions independently, we described the results based on this supermatrix only. As the topology was similar in ML and BI trees, we detailed the species relationships from BI results only. The three individuals of *N. gandarela* formed a well-supported clade in all obtained trees.

The *Nicotiana* species analyzed formed a strongly supported clade (PP = 1.00), and several subclades corresponded to the accepted sections for the genus (Fig. 1). The analysis confirmed the monophyly of the Alatae (PP = 0.99), which is strongly supported as the sister of the *Nicotiana* sect. *Suaveolens* Goodsp. (PP = 1.00). The two newly sequenced species, *N. gandarela* and *N. mutabilis*, were resolved as members of the Alatae. *Nicotiana gandarela* was recovered with high support (PP = 0.99) as sister to the clade with the remaining species in the Alatae, whereas *N. mutabilis* was resolved as closely related to *N. forgetiana* and *N. bonariensis* (PP = 1.00). Similar to previous analyses, low support was observed for the 2n = 18 group and for the relationship of *N. bonariensis* to *N. langsdorffii* (PP = 0.66).

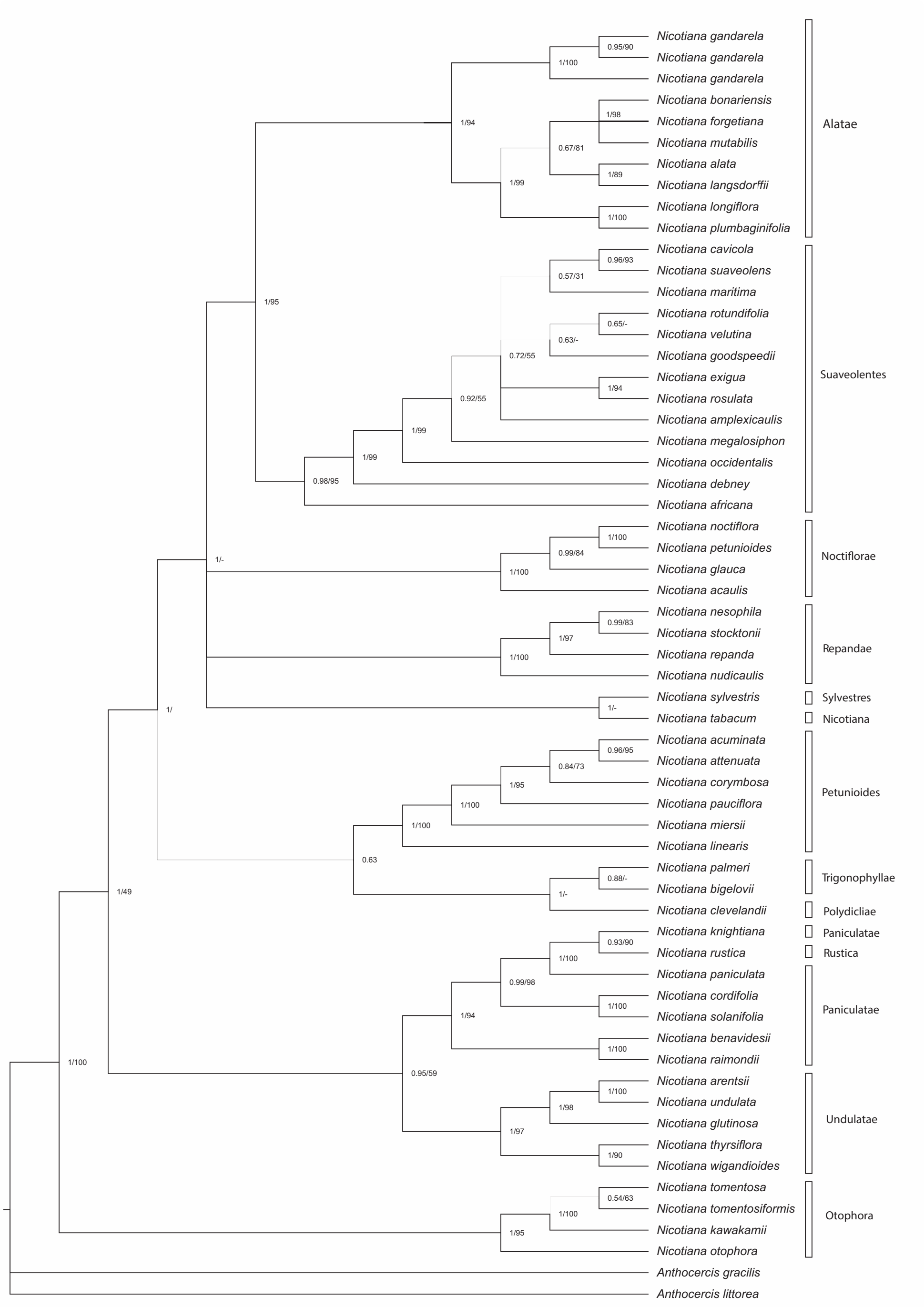

Figure 1. Bayesian 50% majority-rule consensus tree of 56 species of *Nicotiana* L. of the combined nuclear (ITS) and plastid (*ndhF*, *trnLF*, and *trnSG*) datasets. Numbers next to the nodes represent the posterior probabilities (PP) and bootstrap values. Section names (according to Knapp et al. 2004) are indicated on the right. Width of lines defined by PP values.

Taxonomic treatment

***Nicotiana gandarela* Augsten & Stehmann, sp. nov.**
urn:lsid:ipni.org:names:77260722-1
Fig. 2

Type. Brazil. Minas Gerais. Santa Bárbara, André do Mato Dentro, 19°59'43"S, 43°38'39"W, 17 Apr 2018 (fl, fr), *M. Augsten & J.R. Stehmann* 1078 (holotype: BHCB acc.#190958 [BHCB190958!]; isotypes: ICN, RB, to be distributed).

Diagnosis. *Nicotiana gandarela* differs from all other species of the *Nicotiana* sect. *Alatae* by its short corolla tube (< 15 mm), vivid red corolla limb, and unusual shaded, cave-mouth habitat.

Description. Annual herb to 0.5 m high, not branched, pubescent with multicellular, glandular trichomes. Leaves simple, rosulate, crowded, 6–21 cm long, (1.7–) 3.5–5.0 cm wide, persistent, spatulate, slightly discolorous, the blade hirsute throughout with simple, predominantly glandular hairs, long-attenuate at the base, margin sinuous, ciliate, midribs, and secondary veins visible at both surfaces, rounded or obtuse, sometimes acute, at the apex. Inflorescence scapose, paniculate, apical or lateral, composed of monochasial cymes; bracts lanceolate, 4.3 mm long, 1.0 mm wide, viscid-hirsute; pedicels 5.8–7.7 mm long. Calyx hirsute-glandular, deeply lobed, the tube 2.5 mm long, lobes 3–5 mm long, lanceolate, unequal, the apex acute. Corolla aestivation conduplicate, with folded basal petals covering the other three; tube 1.2–1.5 cm long, 3.0–3.4 mm diam., infundibuliform, not inflated at the apex, magenta, trichomes rare, opening ca. 3.4 mm; limb zygomorphic, red, cleft into widely-depressed ovate to very widely ovate lobes, patent or slightly reflexed. Stamens 5, didynamous, adnate ca. 5 mm from the base of the corolla tube, four longer, ca. 12.5 mm long, one shorter, ca. 11.4 mm long, all filaments glabrous; anthers 0.9–1.2 mm long, white, ellipsoid, pollen whitish; nectariferous disk present. Ovary slightly conical, glabrous, style ca. 9.4 mm long, stigma bilobed, green. Capsule 2-valved, included in the calyx, 6.0–7.5 mm long, 4.4–5.5 mm in diameter, ovoid, valves with incised apex. Seeds about 0.7–0.8 mm long, 0.6–6.5 mm wide, subglobose to obovoid, testa foveolate, anticlinal walls sinuous. Chromosome number unknown.

Etymology. The specific epithet "gandarela" is a noun in apposition and refers to Serra do Gandarela, the mountain range complex where this species is found.

Distribution and habitat. The only known population of *Nicotiana gandarela* is at the Serra da Gandarela Mountain range, located in the northeast portion of the Quadrilátero Ferrífero in the Minas Gerais Brazilian State (Fig. 3A). The species occurs in a shaded site placed under a rocky outcrop ledge that extends for about 350 m along a stream and surrounded by a forest matrix, making this area a permanent humid environment. We recognized three subpopulations, each up to 150 individuals, including many seedlings (Fig. 2B). This environment resembles an open cave, and the individuals grow in bare powdery red soil originating from rock decomposition, usually with no other species co-occurring. The habitat is unique, and no similar microhabitat exists in this geologically diverse and complex area (Instituto Prístino: Atlas Digital

Geoambiental: Quadrilátero Ferrífero: Geodiversidade [https://institutopristino.org.br/atlas/quadrilatero-ferrifero/]) Fig. 2A–B.

Phenology. *Nicotiana gandarela* has been collected with flowers and fruits in December and April.

Conservation status. Although the single site for *N. gandarela* would normally suggest the species was Data Deficient (DD), the precarious nature of the region

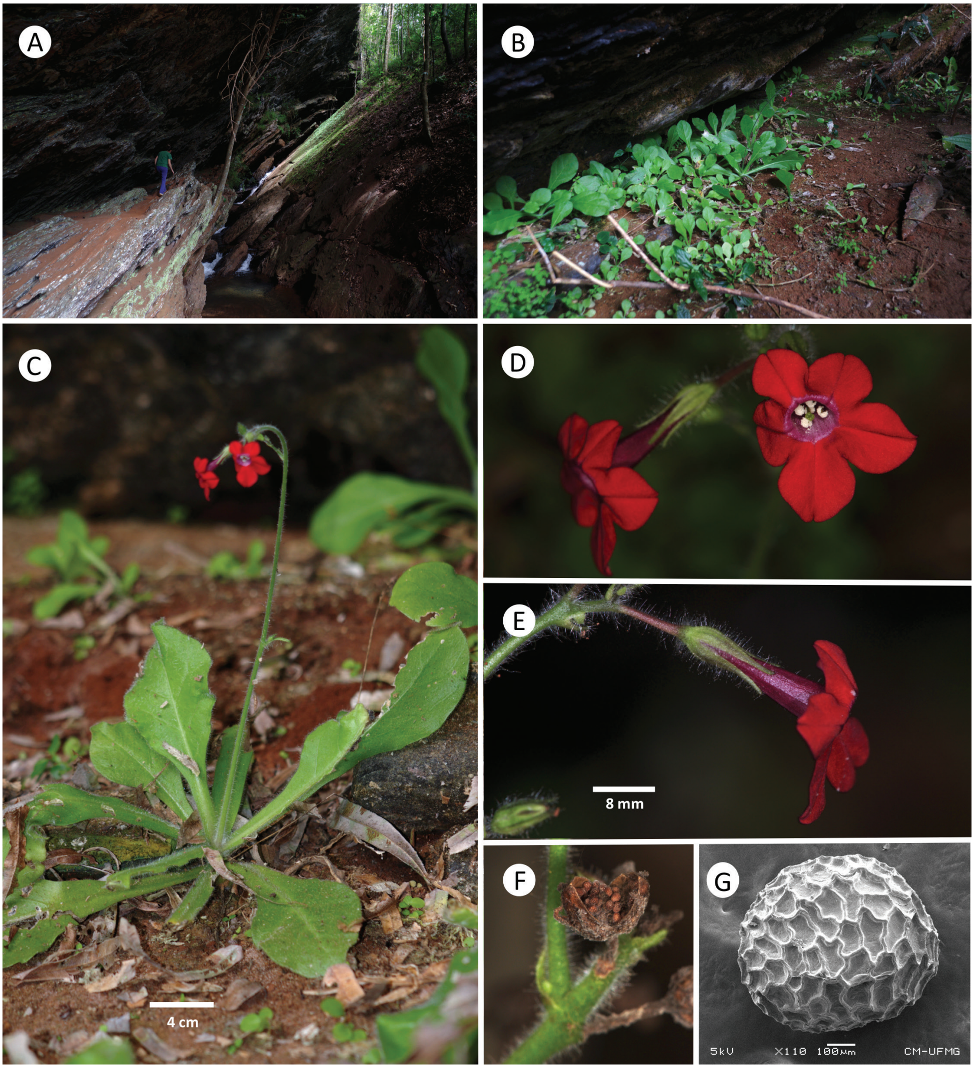

Figure 2. *Nicotiana gandarela* Augsten & Stehmann **A** peculiar habitat of the species, the shaded sites in the base of the shaded ledge **B** seedlings growing in the site **C** habit highlighting the rosulate leaves and the scapose inflorescence **D, E** flowers in lateral and frontal view **F** 2-valvate capsule with many seeds. **G** seed with sinuous anticlinal walls (Scanning Electron Microscopy) **D–G** were obtained from plants of the type population (Augsten and Stehmann 1078, BHCB). Photos by JR Stehmann.

in which it grows leads us to assess it more formally. The species was preliminarily assessed as Critically Endangered – CR (B1, B2a, biii, D), mainly due to its geographic range, as the extent of occurrence (EOO) and the area of occupancy (AOO) are smaller than 100 km^2 and 10 km^2, respectively. The only populations of *N. gandarela* are located on private property and not inserted in any integral conservation unit. In addition, the Quadrilátero Ferrifero suffers from constant habitat loss (Salles et al. 2018) as well as strong pressures from the mining sector (Sonter et al. 2014) with concessions required to explore iron and gold (Instituto Prístino: Atlas Digital Geoambiental 2021 [https://institutopristino.org.br/atlas/quadrilatero-ferrifero/]) (Fig. 3B).

Additional specimen examined. Brazil. **Minas Gerais:** Santa Bárbara. André do Mato Dentro, trilha para a cachoeira, 31 Dec 2017 (fl.,fr.), *D.M.G. Oliveira, P.L.Viana & N.O.Mota 359* (BHCB).

Identification key to the Brazilian species of *Nicotiana* sect. *Alatae*

1 Calyx length equal to the corolla tube.... ***N. azambujae*** **L.B. Sm. & Downs**

– Calyx length shorter than the corolla tube **2**

2 Corolla yellow-greenish or light green, actinomorphic, limb almost entire (shortly lobed), pollen blue .... ***N. langsdorffii*** **Weinm.**

– Corolla white, pink, magenta, purple or red, zygomorphic, limb clearly lobed; pollen green, white or yellow **3**

3 Corolla tube > 30 mm long, limb usually white, filaments adnate to the upper portion of the corolla **4**

– Corolla tube usually < 30 mm long, limb rarely white, filaments adnate to the lower portion of the corolla tube **5**

4 Leaves decurrent, limb strongly zygomorphic, stamens inserted in two levels (4 higher and 1 lower) at the corolla tube, filaments all geniculate .... ***N. alata*** **Link & Otto**

– Leaves auriculate, not decurrent, limb weakly zygomorphic, stamens inserted in three levels (2 higher, 2 middle, and 1 lower) at the corolla tube, filaments not geniculate .... ***N. longiflora*** **Cav.**

5 Corolla changing from white to pink or magenta during the anthesis .... ***N. mutabilis*** **Stehmann & Semir**

– Corolla keeping the same color during the anthesis **6**

6 Leaves evenly distributed and decurrent along the stem; calyx usually > 9 mm; corolla tube usually > 20 mm long, magenta or purple-red .... ***N. forgetiana*** **Hemsl.**

– Leaves usually rosulate, not decurrent on the stem; calyx usually < 9 mm long; corolla tube < 20 mm long, limb red or white **7**

7 Corolla tubular, distally ventricose; limb white .... ***N. bonariensis*** **Lehm.**

– Corolla tube gradually enlarged to the apex, not ventricose, limb red .... ***N. gandarela*** **Augsten & Stehmann**

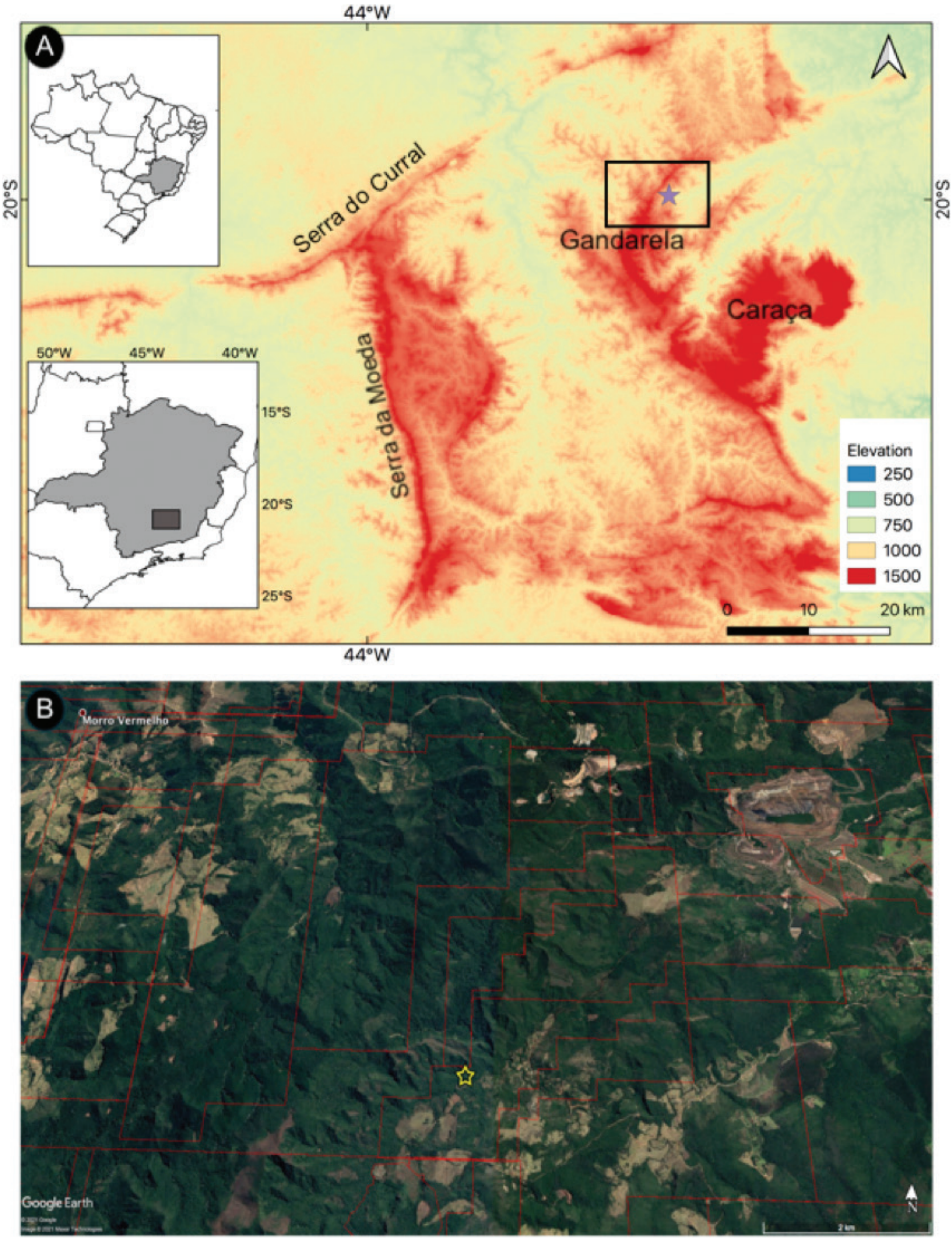

Figure 3. A main mountains of the Quadrilátero Ferrífero in Minas Gerais. Star indicates the *Nicotiana gandarela* Augsten & Stehmann occurrence site **B** Google Earth image showing the regional landscape associated with the *N. gandarela* site, the forest matrix impacted by a small village, and open mining. The red lines indicate mining concessions (Instituto Prístino: Atlas Digital Geoambiental [https://institutopristino.org.br/atlas/]).

Discussion

Overall, the recovered phylogenetic relationships were similar to those previously obtained (Chase et al. 2003; Clarkson et al. 2004; Knapp et al. 2004; Clarkson et al. 2017). Phylogenetic trees generated showed that the samples collected in the Serra da Gandarela in Minas Gerais are genetically distinct from all known species of *Nicotiana* used in our analyses. *Nicotiana gandarela* was recovered as the sister of all other species belonging to the Alatae (both of the groups with distinct cytotypes, 2n = 18 and 2n = 20; Clarkson et al. 2004; Knapp et al. 2004). The internal nodes of the group 2n = 18 showed low support, and the lack of chromosome accounts to the new species does not permit any inferences about the phylogenetic relationships within the section.

The new species is an annual plant, with rosulate leaves, long-attenuate to the base, sessile, viscid-pubescent, and a zygomorphic corolla, all morphological traits associated with the species of the Alatae (Knapp et al. 2004). The decurrent leaf bases on the stem and the abrupt dilatation at the corolla throat, commonly found in the other species of the section (Goodspeed 1954), are lacking in the new species. The habit resembles that of *N. bonariensis* because of its rosulate leaves and scapose inflorescence. However, *N. gandarela* differs from *N. bonariensis* in its ombrophilous and extremely narrowly distributed habitat, its diurnal anthesis with red corollas with tube < 15 mm long, rather than being being heliophilous, widespread, with nocturnal anthesis, and white corollas with a tube usually > 15 mm long.

Corolla color is variable in the Alatae, including white, yellow, pink, magenta, and purple-red. Such variation reflects an evolutionary history of radiation to distinct pollinator agents (Ippolito et al. 2004; Kaczorowski et al. 2005; Knapp 2010). Red flowers are usually associated with bird pollination (Faegri and van der Pijl 1979), and hummingbirds were already reported pollinating *N. forgetiana*, a pink to purple-red flowered species distributed in southern Brazil (Ippolito et al. 2004). Empirical data on the effective pollinator of *N. gandarela* are still necessary.

The Quadrilátero Ferrífero in Minas Gerais is located in the southern Espinhaço Range, a mountainous chain where the Cerrado (tropical Savannah) and Brazilian Atlantic Forest, two hotspots of biodiversity, connect (Mittermeier et al. 2004). The vegetation is a mosaic of topologies and vegetation, including grasslands, savannah, and forests (Spósito and Stehmann 2006), whose distributions are influenced by an altitudinal gradient, ranging from about 700 m to 2,080 m (Borges et al. 2011). In the region, besides the new species, a further two species of the Alatae are found, *N. bonariensis* and *N. langsdorffii*. The first usually inhabits the grasslands at higher altitudes known as Campos Rupestres (Silveira et al. 2016), while the second is generally associated with disturbed areas. Both grow mainly in open and sunny sites, as with most species of the Alatae, and the peculiar, shaded habitat of *N. gandarela* seems to be unique in the section. The microhabitat of the new species roughly resembles that of *Petunia exserta* Stehmann, an endemic species from Rio Grande do Sul, in

Southern Brazil, but where the geology and vegetational matrix are totally different (Lorenz-Lemke et al. 2006; Stehmann et al. 2009). In both cases, few or no species share the ground where these two species grow, meaning that they are fragile, with low, competitive capacity, but are presumably adapted to survive in these empty niches. Penumbral plant communities have also been described for the Campos Rupestres in Minas Gerais, where several species were reported as growing in small caves (Alves and Kolbek 1993), but until now, no Solanaceae species have been registered in these areas.

The new species deserves special conservation attention because it inhabits a small and rare habitat, if not unique, in the Quadrilátero Ferrífero, a region that is suffering intensive habitat loss (Salles et al. 2018) and has been the scenario of recurrent environmental disasters (Carmo et al. 2017; Rotta et al. 2019). Floristic inventories looking for new populations should be carried out in the surrounding forested areas, including the Serra do Gandarela National Park, following the drainage lines in the valleys. Also, we suggest the engagement of local people in trying to find other populations because of the impossibility of looking for similar areas since they are hidden by forest in satellite images. In the end, although we emphasize that the species should be primarily preserved in its natural habitat, ex-situ conservation measures might also be necessary (CNCFlora 2016) in order to prevent its extinction.

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Supplementary material 1

Table S1

Authors: Mariana Augsten, Pablo Burkowski Meyer, Loreta B. Freitas, João A. N. Batista, João Renato Stehmann

Data type: GenBank accesions

Explanation note: Genbank accessions. ITS sequences from Chase et al. 2003, Plastidial sequences (*trnL-F*, *trnS-G* and *ndhF*) from Clarkson et al. 2004. Sequences from this work marked with an * (*Nicotiana gandarela* and *Nicotiana mutabilis*).

Link: https://doi.org/10.3897/phytokeys.190.76111.suppl1

Supplementary material 2

Figure S1

Authors: Mariana Augsten, Pablo Burkowski Meyer, Loreta B. Freitas, João A. N. Batista, João Renato Stehmann

Data type: Phylogenetic

Explanation note: Bayesian 50% majority-rule consensus tree of 56 species of *Nicotiana* L. of the nuclear (ITS) dataset. Numbers next to the nodes represent the posterior probabilities (PP).

Link: https://doi.org/10.3897/phytokeys.190.76111.suppl2

Supplementary material 3

Figure S2

Authors: Mariana Augsten, Pablo Burkowski Meyer, Loreta B. Freitas, João A. N. Batista, João Renato Stehmann

Data type: Phylogenetic

Explanation note: Bayesian 50% majority-rule consensus tree of 56 species of *Nicotiana* L. of the plastid (*trnL-F*, *trnS-G* and *ndhF*) datasets. Numbers next to the nodes represent the posterior probabilities (PP).

Link: https://doi.org/10.3897/phytokeys.190.76111.suppl3

CAPÍTULO 2. Molecular and ecological evidence reveal a diversification process in the Nicotiana sect. Alatae (Solanaceae) throughout the escarpments of the southern Brazilian highland plateau

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Molecular and ecological evidence reveal a diversification process in the *Nicotiana* sect. *Alatae* (Solanaceae) throughout the escarpments of the southern Brazilian highland plateau

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ABSTRACT

Integrative taxonomy employs several biological information sources and techniques to enhance the precision of determining taxa boundaries. In this study, we have applied multiple approaches to revisit *Nicotiana forgetiana* s.l. (Solanaceae), a tobacco with magenta flowers found in the southern Brazilian plateau, previously identified in other studies as encompassing a species complex. We used molecular markers to evaluate the genetic diversity based on plastid DNA and modelled the ecological niche, mapping current and historical distribution of taxa. Our findings indicate the existence of two monophyletic groups with robust support as previously indicated by morphological measures, wherein individuals in each group did not share haplotypes and there is no ecological niche overlap between groups. In accordance with previous morphometric studies, we confirmed that the populations located at the borders of the highlands in Santa Catarina and north-eastern Rio Grande do Sul Brazilian states represent a distinct species. Furthermore, our study offers evidence that the southern region of Brazil was the stage of an important diversification process for *Nicotiana* sect. *Alatae*.

Keywords:*Nicotiana forgetiana*, "*Nicotiana forgetiana* morphotype rastroensis", *Nicotiana* sect. *Alatae*, tobacco, species complex

INTRODUCTION

Solanaceae is a recognised plant family with diverse uses for human beings, such as sources of food, drugs, insecticides, and ornamental plants, resulting in a global market worth billions of dollars (Hawkes, 1999; Samuels, 2015). *Nicotiana* L., a genus to which tobacco belongs, is one of the most extensively worldwide utilised plants, with ancient use dating back to 12,000 years ago (Duke *et al.*, 2021). The species *N. tabacum* L. and *N. rustica* L. are widely cultivated due to their economic importance, leading to significant investments in research on this genus. Moreover, *Nicotiana* has been used in genetic studies since the 1930s (e.g., Brieger, 1935; Shinozaki *et al.* 1986; McClure *et al.* 1990; Sierro *et al.* 2014) and continues to be relevant in current research on new technologies (Gebhardt, 2016). On the other hand, such studies have focused preferentially on a few species, which has resulted in neglecting different lineages and wild species in the genus.

Nicotiana belongs to the Nicotianoideae clade, which is closely related to the Solanoideae clade that, together, form the well-known "x = 12 clade" (Olmstead *et al.*, 1999; Särkinen *et al.*, 2013). With almost 100 species, *Nicotiana* is the fifth largest genus in the Solanaceae family (Goodspeed, 1954; Knapp, 2020) and recent discoveries have expanded our knowledge on the genus diversity. Several new species have been described recently in Australia (Chase *et al.*, 2018), Chile (Santilli *et al.*, 2021), and Brazil (Stehmann, Semir, & Ippolito, 2002; Augsten *et al.*, 2022). In addition, a species previously believed to be extinct has been rediscovered (Funez *et al.*, 2023). These findings highlight the importance of continuous researches and conservation efforts to understand and protect this fascinating and diverse genus.

A notable degree of chromosome diversity is found in *Nicotiana*, which is characterised by several allopolyploid species and a varied counts (Goodspeed, 1954; Knapp, Chase,

& Clarkson, 2004). Notwithstanding these variations, the genus has upheld a consistent circumscription since the publication of the seminal monograph by Goodspeed (1954), wherein an extensive analysis of species morphology and cytology was conducted, leading to inferences regarding the natural history of the genus (Knapp, Chase & Clarkson, 2004). However, at the turn of the millennium, molecular evidence was introduced, which clarified the internal relationships and dated the events of diversification (Chase, 2003; Clarkson *et al.*, 2004; Clarkson, Dodsworth, & Chase, 2017). Currently, there are 13 accepted sections in *Nicotiana*, most matching with the circumscription coming from Goodspeed's revision (Knapp *et al.*, 2004).

Although having a wide geographical distribution, occurring in the Americas, Australia and Africa (Goodspeed, 1954; Chase, 2003), the evolutionary history of *Nicotiana* seems to be linked with South American geomorphological history, more specifically to the Andean region, where several sections and lineages are distributed, such as *N.* sect. *Nicotiana*, *N.* sect. *Noctiflorae* Goodsp., *N.* sect. *Paniculatae* Goodsp., *N.* sect. *Petunioides* G. Don, *N.* sect. *Sylvestres* S. Knapp, *N.* sect. *Tomentosae* Goodsp., and *N.* sect. *Undulatae* Goodsp. (Knapp *et al.*, 2004). Only *N.* sect. *Alatae* Goodsp. has most of its species with distribution restricted to Southeast and South Brazil, Uruguay and Argentina, near to the Atlantic coast (Goodspeed, 1954; Knapp *et al.*, 2004). Diversification of the *N.* sect. *Alatae* was estimated respectively in 6.2 Mya and 7.7 Mya, according to the plastid or nuclear markers used for the reconstruction (Clarkson *et al.*, 2017), before the uplifting of the southern Andean region (Boschman & Condamine, 2022).

The species in *N.* sect. *Alatae* are all easily recognised by the herbaceous habit, rosulate and sessile leaves, viscid pubescence, few leaves on upper stems, and corolla tube distally inflated (Knapp *et al.*, 2004). Additionally, this section is divided in two main groups of species with different chromosome numbers, the first with two self-compatible, white-flowered and n = 10 chromosomes species, and the second with seven species with n = 9 chromosomes, predominantly self-incompatible and displaying flowers with varied shape, size, and colours (Lee *et al.*, 2008). However, the internal phylogenetic relationships in the *N.* sect. *Alatae* are not resolved, and more studies are in need (Clarkson *et al.*, 2004, 2017; Augsten *et al.*, 2022).

The group of species with a chromosome number of n = 9 seems to have diversified in southern Brazil, in a region characterised by a transition zone where Pampa and Brazilian Atlantic Forest biomes meet, along with the presence of subtropical highland grasslands, which harbour a variety of endemic species (Iganci *et al.*, 2011). Many authors have suggested that Quaternary climate changes have influenced speciation in this region, especially for plant groups with a recent diversification, such as *Adesmia* DC (Fabaceae) and *Calibrachoa* Cerv. and *Petunia* Juss. (Solanaceae) (Fregonezi *et al.*, 2013; Iganci *et al.*, 2013; Mäder *et al.*, 2013; Turchetto *et al.*, 2014; Barros *et al.*, 2015).

One species in the *N.* sect. *Alatae*, *Nicotiana forgetiana* Hemsl., has recently gathered attention due to a divergent taxonomic circumscription. This magenta-flowered *Nicotiana* was first described by Hemsley in 1904 from seeds collected by Mr Forget in southern Brazil and is one of the parents of the famous hybrid *N. x sandarae*, commercially used for ornamental purposes (Fig 1: A, B). Ecological data (Kazorowski, Gardener & Holtsford, 2005) suggested that populations from the canyon regions in Santa Catarina Brazilian state in the Serra Geral could be a new species, once they sjowed a shorter tube and differing nectar composition, and presented the name *Nicotiana forgetiana* morphotype rastroensis (Fig 1: C, D). Floral morphometry from several individuals corroborated the separation between the two groups, considering the size of corolla tube (Teixeira *et al.*, 2022).

In the present study, we adopted an integrative approach (Dayrat, 2005; Knowles & Carstens, 2007) to revisit *Nicotiana forgetiana* s.l. First, we expanded our sampling to cover the entire occurrence area and used molecular markers to assess the phylogenetic relationships between the two putative groups. Additionally, we analysed the ecological niche of each group, mapped their current distribution, and modelled their distribution in two past times to gain insights on the historical processes associated with Quaternary climate changes in southern Brazil, as observed in other taxonomic groups.

Figure 1. General view of *Nicotiana forgetiana,* “*N. forgetiana* morphotype rastroensis” and their occurrence environment: adult individual in nature (A, B – *N. forgetiana*; C, D – “*N. forgetiana* morphotype rastroensis”); flower in frontal view (B – *N. forgetiana*; D – “N. rastroensis).

MATERIAL AND METHODS

Study area

The study area corresponds to that of *N. forgetiana* s.l. occurrence in southern Brazil, from Rio Grande do Sul to Santa Catarina Brazilian states. It is specifically associated to the clime type Cfa, characterised by a temperate climate without a dry season and a warm summer. In this region, the temperature of the coldest month ranges between zero and 18ºC. Additionally, the area experiences a warm summer, with the temperature of the hottest month exceeding 22ºC (Peel, Finlayson, & McMahon, 2007) The elevation ranges from 600 m to ~ 1,800 m above sea level.

The distribution of the species is linked to a landscape where forests and grasslands are intermixed (IBGE, 2012). The species' primary habitat is the rocky outcrops associated with river basin valleys or escarpments at the highland plateau border.

Sampling and data collection

We sampled young and health leaves from adult plants during the springs in 2018 and 2019, from September to December (Fig. 2; Supporting Information, Table S1). Sampling covered the entire occurrence area, reflecting the natural distribution and abundance of each putative taxon. We collected "N. rastroensis" (three sites, 12 individuals) and *N. forgetiana* (seven locations, 44 individuals) samples, totalising 56 individuals (Table S1). Form each site (hereafter population), we took the geographical coordinates and collected one voucher, which were deposited at BHCB (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) or ICN (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) herbaria.

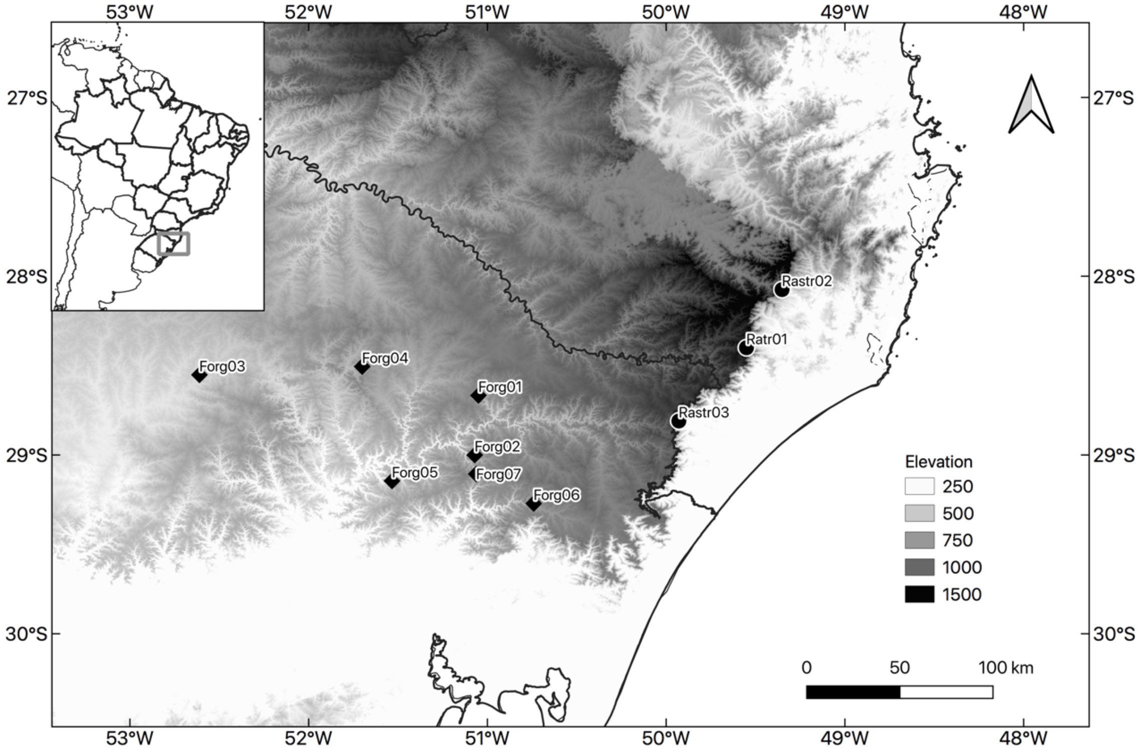

Figure 2. Collection sites for *Nicotiana forgetiana* and “Nicotiana rastroensis”. Population codes follow Supporting information Table S1.

DNA extraction

We collected and dried leaves in silica gel and mechanically pulverised them using liquid Nitrogen. We extracted the total genomic DNA following a CTAB (cetyl-trimethyl ammonium bromide; Sigma-Aldrich Chem. Co., St. Louis, USA)-based method (Roy *et al.*, 1992). We measured DNA concentration with a Qubit Fluorometer (Thermo Fisher Scientific Co., Waltham, USA) and DNA quality with a NanoDrop DN-1000 Spectrophotometer (Thermo Fischer). All samples were diluted to uniform concentration and stored at -20 °C until use.

Amplification and sequencing of chloroplast markers

We amplified the noncoding plastid intergenic spacers *trnS-trnG*, *rps12-rpl20*, and *rpl32-trnL* using the universal primers (Hamilton, 1999; Shaw *et al.*, 2005, 2007). These regions were informative for intraspecific analyses in Solanaceae (e.g., Lorenz-Lemke *et al.*, 2010; Fregonezi *et al.*, 2013; Longo *et al.*, 2014; Turchetto *et al.*, 2014). PCR conditions followed Mäder & Freitas (2019)'s protocols. These spacers belong to the Large Single Copy (*trnS-trnG* and *rps12-rpl20*) and Small Single Copy (*rpl32-trnL*) regions of the chloroplast, which are recognised as the regions where highly variable sequences occur (Wang *et al.*, 2022). Amplicons were purified using 20% polyethylene glycol (Dun & Blattner, 1987) and sequenced on demand. The sequences will be deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Genetic diversity and phylogenetic analysis

We aligned the DNA sequences using MEGA11 software (Tamura, Stecher, & Kumar, 2021) with the ClustalW algorithm and manually edited them when necessary. Therefore, we removed the poly-T/A regions from the analyses because their homologies cannot be adequately accessed (Aldrich, Cherney, & Merlin, 1988; Lorenz-Lemke *et al.*, 2010). In addition, we concatenated the three plastid sequences in Mesquite (Maddison, W. P. & Maddison., 2019) and used this matrix as input for the remaining analyses.

We determined the haplotypes using DNASP v5.10.01 (Rozas *et al.*, 2003) and estimated the basic descriptive molecular diversity statistics, such as haplotype and nucleotide diversity, and the neutrality tests Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) in ARLEQUIN v3.5.1.2 (Excoffier & Lischer, 2010). In addition, we estimated the haplotypes' evolutionary relationships with NETWORK v4.600 (available at http://www.fluxux-engineering.com) using the median-joining network method (Bandelt, Forster, & Rohl, 1999).

The phylogenetic relationships among the 56 sampled individuals were verified through a Bayesian inference (BI) using BEAST v1.10.4 (Suchard *et al.*, 2018). As outgroup we used sequences of whole chloroplast genome available ate Genbank. Those are: *N. plumbaginifolia* (GenBank accession: LC649170.1) and *N. glauca* (NC_056979.1). We used the following parameters in BI: substitution model, GTR; site heterogeneity model, Gamma + Invariant Sites; strict clock; tree prior model; speciation: birth-death process; length of chains, 10 million sampled each 1000. The selection of the GTR model was based on the JModel test v3.06 (Darriba *et al.*, 2012) and the selection of the richest parameter (Abadi *et al.*, 2019).

To estimate the divergence time between *N. forgetiana* and "N. rastroensis" individuals, we ran the same parameters listed above with one representative sequence per haplotype. We used the clock rate previously published for herbaceous Solanaceae plants (A). Clades with >0.95 posterior probability were considered well-supported.

Changes in population size over time for each putative species were estimated with a Bayesian skyline plot analysis (BSP; Drummond *et al.* 2005) also performed in BEAST (Suchard *et al.*, 2018) and following the same parameters listed above. To reach a good MCMC convergence, we ran for 100 million chains sampled every 1000.

We used Tracer v1.7.1 (Rambaut *et al.*, 2018) to evaluate the effective sample size (ESS > 200) values of log files output, and TreeAnnotator v1.10.4 to construct a consensus tree using a 10% burn-in. We visualised and edited phylogenetic trees with FigTree v1.4.4. Inkscape software was used to edit tree images, at last. The pairwise p-distance was calculated in MEGA11. This distance is the proportion (*p*) of nucleotide sites at which two sequences being compared are different. Therefore, we choose a boxplot to visually demonstrate the p-values differences between putative species and withing them.

Ecological niche modelling analyses

To investigate the existence of suitable common habitats between the putative species, we performed an Ecological Niche Modelling (ENM) analysis considering present and two past climate conditions. Bioclimatic variables were downloaded from WorldClim (www.worldclim.org). Our models included present variables (1970–2000) and two past scenarios (Last Glacial Maximum - LGM - *ca.* 21 kya; and Last Interglacial - LIG - *ca.* 120–140 kya). For occurrence data, we downloaded information from INCT – Virtual Herbarium of Flora and Fungi (speciesLink) records described for *N. forgetiana,* as "N. rastroensis" is yet under description process. Data were manually checked, attributed for each putative species according to their geographical occurrence, and filtered for only trustable determination and coordinates accuracy. We cropped the bioclimatic layers using QGIS, adjusting to the studied area. We estimated correlations between layers in ENMTools v1.0 (Warren *et al.*, 2021) in R. We selected three non-correlated variables that also we think to be relevant to implement models: BIO4 (Temperature Seasonality - standard deviation ×100), BIO6 (Minimum Temperature of Coldest Month), and BIO12 (Annual Precipitation). The maximum entropy algorithm was used in MAXENT v3.4.1 (Phillips, Anderson, & Schapire, 2006) to estimate species' ecological niches. We used auto features with five and ten cross-validation replicates for "N. rastroensis" and *N. forgetiana*, respectively, with 1000 iterations, 10000 background points in each run, and logistic output (Phillips & Dudík, 2008). We determined area for niche modelling latitudes from Niche overlap as measured in ENMTools v1.0, from Schöner`s D, I, and

Rank Values. Also, an identity test was performed to obtain the statistical significance of niche overlap (Warren *et al.*, 2021).

RESULTS

Haplotypes and genetic diversity

The plastid intergenic spacers *trnS-trnG*, *rps12-rpl20* and *rpl32-trnL* resulted in an alignment with 2,475 base pair (bp) long. The alignment size of each region, percentage of GC, number of variable sites and percentage of informative sites are described in Table 1. The *trnS-trnG* was the most informative region for *N. forgetiana,* whereas *rpl32-trnL* was for “N. rastroensis”. The percentage of informative sites ranged from 0 to 0.77%. This alignment produced seven haplotypes for *N. forgetiana* and three for “N. rastroensis”; haplotype and nucleotide diversities are listed in the table (Table 2). Tajima’s D and Fu’s F were negative and nonsignificant for *N. forgetiana* and positive and nonsignificant for “N. rastroensis” (Table 2). There was no haplotype sharing between the two putative species (Supporting Information, Fig. S1; Table S1). Each population of “N. rastroensis” presented exclusive haplotypes. The genetic distance measured by p-values of intraspecific sequences was shorter than the interspecific comparisons (Supporting Information, Fig S2). Intraspecific distance was low, sometimes zero, indicating low or no variation between individuals of the same putative species.

Table 1. Alignment information based on three plastid intergenic spacers sequences for *Nicotiana forgetiana* and “N. rastroensis” .

Taxa	Molecular marker	Alignment length (bp)	%GC	V	I (%)
N. forgetiana	*trnS-trnG*	648	30.6	5	0.77
	rps12-rpl20	791	33.3	2	0.25
	rpl32-trnL	1,036	28.7	3	0.28
	Total	2,475	30.5	10	0.40
“N. rastroensis”	*trnS-trnG*	648	30.6	0	0
	rps12-rpl20	791	33.3	3	0.37
	rpl32-trnL	1,036	28.6	2	0.19
	Total	2,475	30.5	5	0.20

V - number of variable sites; I(%) – proportion of parsimoniously informative sites

Table 2. Diversity indices and neutrality tests for *N. forgetiana* and “N. rastroensis” based on plastid markers.

Taxa	N	H	h (SD)	□ % (SD)	Tajima’s D	Fu’s Fs
N. forgetiana	44	7	0.75 (0.04)	0.05 (0.04)	-1.315	-1.347
“N. rastroensis”	12	3	0.71 (0.07)	0.05 (0.04)	1.022	1.250

N – sample size; H – number of haplotypes; h – haplotype diversity; □ – nucleotide diversity;

<u>Evolutionary relationships between haplotypes</u>

In the BI phylogenetic analysis (Fig. 3), we observed two fully supported main clades (PP = 1), each encompassing haplotypes of one putative species. The outgroup *N. plumbaginifolia* appeared as the sister species of *N. forgetiana* – “N. rastroensis” clade with high support (PP = 0.99). The estimated divergence time between *N. forgetiana* and “N. rastroensis” was ~100 kya, whereas their divergence from *N. plumbaginifolia* occurred at ~175 kya (Supporting Information, Fig S3). *Nicotina glauca* that belongs to *N.* sect. *Noctiflora* appeared as sister group to remained individuals. Due to the evolutionary distance and lack intermediary representants for other sections, we did not estimate the divergence time between *N. glauca* and *N.* sect. *Alatae*.

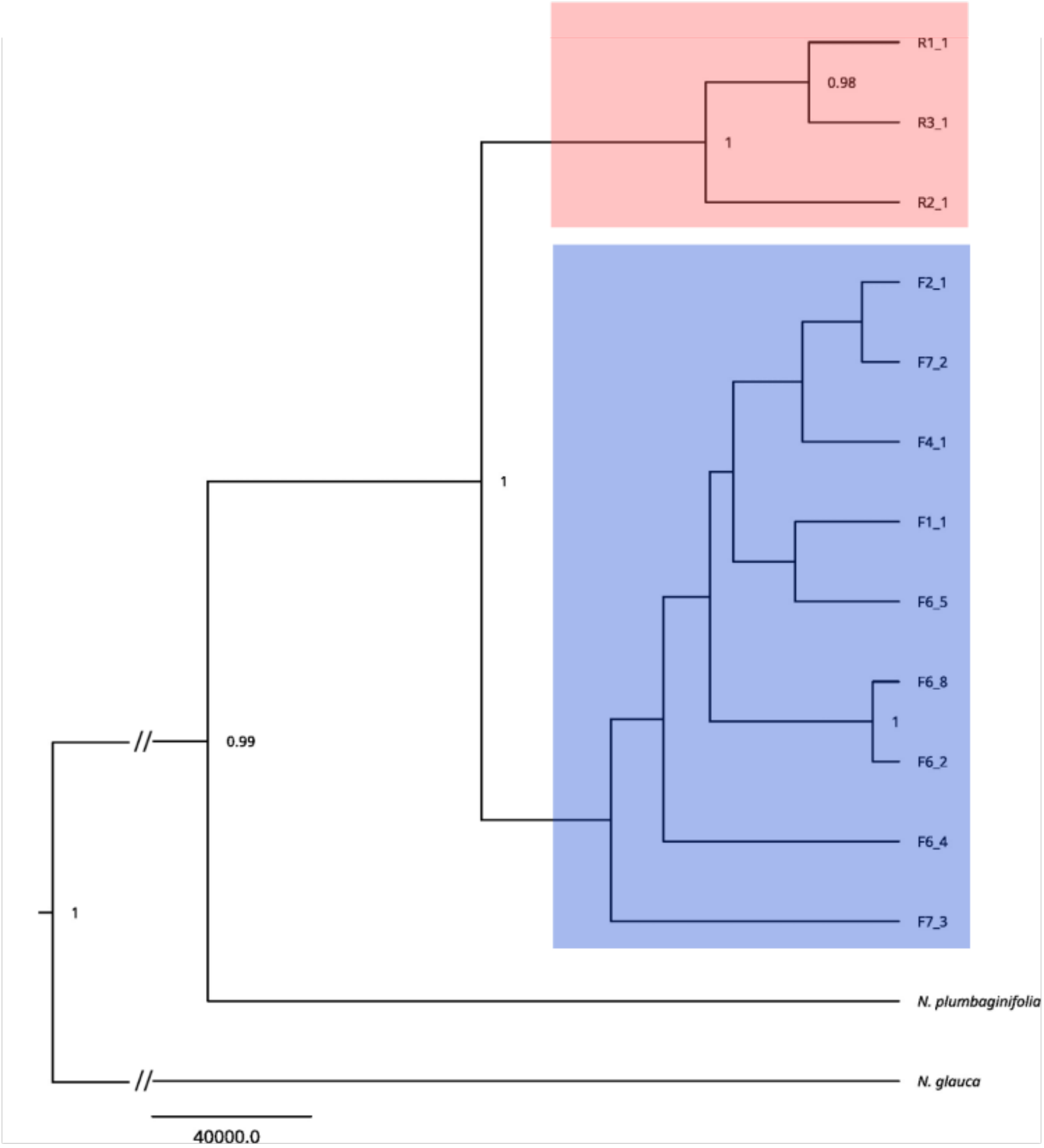

Figure 3. Bayesian inference based on one sequence per haplotype. F represents *N. forgetiana* and R "N. rastroensis" followed by numbers indicating population and individual codes, respectively. Values beside branches indicate support (only PP ≥ 0.98 were shown).

Ecological suitability, niche specificity, and effective population size changes over time

ENM (Supporting Information, Fig. S5) resulted in AUC > 0.95, which indicates models are suitable. In general, models recovered the current occurrence area of both entities, extrapolating a bit corresponding to the occurrence of subtropical mixed forest. For "N. rastroensis", current suitable areas reached the high mountains at the south of Minas Gerais, as Serra da Mantiqueira, where there is no record for the taxon. In the past models, we observed low differences for "N. rastroensis" potential distribution. For *N. forgetiana,* the forecasted LGM suitable area was quite the same to the current distribution, whereas that predicted to LIG was a little larger. In agreement with ENM and neutrality tests, the Bayesian skyline analysis showed demographic stability over time, with no drastic changes in *Ne* (Supporting Information, Fig. S5). According to niche overlap tests, species did not share the same niche, in fact species differed in niche occurrence. Niche overlap scores were Schoener's $D = 0.18$, $I = 0.40$, and RR =- 0.80, all significantly lower than the null model ($p < 0.03$).

DISCUSSION

Our findings showed that the magenta *Nicotiana* with distribution in the highlands of southern Brazil comprises two genetically and ecologically distinct groups, and must be accepted as two different species (De Queiroz, 2007). Two monophyletic groups were recovered with high support, populations did not share haplotypes, and there is no ecological niche overlap, confirmed by the distribution models for current distribution and potential past suitability. Despite the phenotypic similarities, floral morphological differences tested by geometric morphometric analysis already suggested the separation in two groups (Teixeira *et al.*, 2022). Based on the integrative approach, which combines different and complementary evidence (Dayrat, 2005; Knowles & Carstens, 2007), we propose to recognise the populations from the highland border in Santa Catarina and north-eastern Rio Grande do Sul Brazilian states as a distinct species from those individuals that occur in the interior of the Rio Grande do Sul and Santa Catarina. The size of the corolla tube is the main diagnostic morphological character. While "N. rastroensis" (under description) measures below 25 mm long, *N. forgetiana* presents longer flowers, with corolla tube measuring usually 25 to 35 mm long (Augsten, unpubl. data).

Phylogenetic studies had provided insights to recognize cryptic species in Solanaceae, where the morphological similarity hid different evolutionary histories. In Australian Nicotianas (*N.* sect. *Suaveolentes* Goodsp.) molecular phylogenies were essential to identify cryptic species or to confirm previous assumptions due to morphological variations, leading to recognition of new taxa (Chase *et al.*, 2018). This methodology was also important to elucidate the *Nicotiana benthamiana* species complex, that did not comprehend one only taxa, as formerly thought, but in true five species, with morphological and geographical differences (Cauz-Santos *et al.*, 2022; Chase *et al.*, 2022). The authors reinforce the importance of this reciprocal illumination when comparing different sets of data to identify and describe these species (Chase *et al.*, 2021). In the Neotropical genus *Brunfelsia*, phylogenetic studies revealed that one taxon, *B.*

uniflora, would be paraphyletic, as accessions from different localities appeared in different clades of South America group. This led to extensive revision of collections, revealing morphological differences between them, leading to recognition of *B. plowmaniana*, which also has a different geographic distribution than *B. uniflora* (Filipowicz, Nee, & Renner, 2012). Furthermore, in *Calibrachoa*, two new species were described based primarily in genetic studies using molecular data that provided evidences for recognizing them as different taxonomic entities (Stehmann, Mäder & Freitas, 2022).

Plastid data showed a high haplotypic and low nucleotide diversities (Table 2). Similar values can be found in other Solanaceae occurring in the region. For example, *Petunia exserta* and *Calibrachoa pygmaea*, species from Pampa`s region, also showed high haplotypic and low nucleotide diversities (Lorenz-Lemke *et al.*, 2006; Mäder *et al.*, 2019), which may be associated with the restricted area of occurrence of the species. High haplotypic and nucleotide diversity values were found in *Petunia* species showing a more extensive range of distribution of the species (Turchetto et al., 2014; Soares, Fagundes, & Freitas, 2023).

“N. rastroensis” and *N. forgetiana* do not share any haplotype (Supporting information, Fig. S1) and haplotypes from “N. rastroensis” derived from the core cluster from *N. forgetiana*. Sharing of haplotypes is common, even in different species, when it comes to phylogenetically close species. That has already been mentioned for *Petunias,* suggesting the persistence of ancestral polymorphisms (Lorenz-Lemke *et al.*, 2010; Longo *et al.*, 2014; Ramos-Fregonezi *et al.*, 2015). Therefore the non-sharing of haplotypes of the *Nicotiana* studied here reinforce the reproductive isolation from both, supporting the hypothesis for recognizing the species and, besides that, may indicate that the separation between them is older than previously believed.

Species of *Nicotiana* are generally associated with open environments (Knapp *et al.*, 2004), and species of *N. sect. Alatae* seem to radiate and occupy habits with distinct environmental conditions, from penumbral sites, such as *N. gandarela* (Augsten et al. 2022), to entirely sunny places, such as *N. bonariensis* (Vignoli-Silva & Mentz 2005). *Nicotiana forgetiana* and *"*N. rastroensis" are species associated with the forest matrix, but usually occurring on rocky outcrops in addition also in disturbed sites as along the roadsides (Smith & Downs, 1966; Vignoli-Silva & Mentz, 2005). The highland plateau in southern Brazil was the stage for speciation of certain groups of plants (Iganci *et al.*, 2011; Fregonezi *et al.*, 2013). During the Glacial Maximum, there was an expansion of grasslands, and retraction of forest areas (Behling, 2002), while after 3,000 ybp, *Araucaria* forest spread on grasslands, with the prevailing wetter and cooler climate (Behling, 1997, 2002; Dümig *et al.*, 2008; Oliveira, Roig, & Pillar, 2010). As mentioned before, ecological niche modelling for both groups of species showed little or almost no change over the evaluated time (Supporting information, Fig. S5), and neutrality tests and the Bayesian skyline analysis indicated demographic stability for the species studied (Table 2, Supporting information, Fig. S4). These findings suggest that climate change possibly have not substantially affected the ranges of the two putative distinct species and we can suppose that the separation between these species may have occurred before these cycles of changes evaluated. Our data showed that *N. forgetiana* and 'N. rastroensis' clades can have diverged *ca*. 100 kya (Supporting information, Fig. S3). However, caution is needed to use such inference as the complete phylogenetic study (Augsten *et al.*, 2022) on *N. sect Alatae* did not include all species of the section nor representatives of the *N. forgetiana* and "N. rastroensis", resulting in an unclear relationship between the two groups studied here.

Due to the fact that these species are associated with rock outcrops, especially in the case of "N. rastroensis", with canyons valleys, these habitats could be characterised as refuges for the isolated populations, which accumulated small morphological changes. Teixeira et al. (2022) found a reduction in suitability areas during the Maximum Glacial for *N. forgetiana*. The discrepant findings could be associated with the different databases that joined *N. forgetiana* and "N. rastroensis" occurrence sites and with the distinct used set of climatic variables. Effective population size also remained stable, and did not indicate any expansion or retraction of those species, which can also corroborate for these suppositions. (Supporting information, Fig. S4). The escarpment areas in the ecotone with the subtropical highland grasslands were already pointed out as areas that remained stable for other genera (Barros *et al.*, 2015). Climatic stable areas can hold a great number of species, especially those of small range occurrence (Morueta-Holme *et al.*, 2013; Enquist *et al.*, 2019).

Pollination in *Nicotiana* is diversified and done by bees, moths, birds, and bats, playing an essential role in the evolutionary history of some lineages (Knapp, 2010). *Nicotiana forgetiana* and "N. rastroensis" have hummingbird pollination syndrome, with tubular, scentless magenta flowers that open during the day, and are sometimes pendulous (Faegri & van der Pijl, 1980). Ornithophily was also reported to three other species of *N. sect. Alatae*: *N. gandarela*, *N. mutabilis,* and N. *langsdorffii* (with pink and white flowers), and have probably independent origins in the section (Stehmann *et al.*, 2002; Kaczorowski, Gardener, & Holtsford, 2005; Augsten *et al.*, 2022). Kaczorowski *et al.* (2005) suggested that nectar traits, instead of floral form, could shape pollinator preferences. The authors reported that the nectar composition in populations corresponding to "N. rastroensis" had characteristics compatible with melittophily, whereas those found in *N. forgetiana* matched with ornithophily. In *Petunia* it was demonstrated that changes in a single gene caused a major shift in pollination biology of species, (Hoballah *et al.*, 2007). *Nicotiana* sect. *Allatae* potential group for studies of the same nature, considering the different pollination syndromes found in this cluster. Also empirical data are lacking to determine the effective pollinator for almost all species in the section not allowing the complete understanding of the pollination shifts in these plants.

Conclusion

Our finds evidences of diversification of *N.* sect *Alatae* in southern Brazilian, region also critical for diversification of other herbaceous Solanaceae, as *Calibrachoa* and *Petunia* (Fregonezi et al., 2013). The presence of morphological discontinuity, revealed through morphometric analysis (Teixeira et al., 2022), in addition to the new evidence indicating that previously grouped populations are genetically distinct and have different ecological preferences, confirms that *N. forgetiana* and "N. rastroensis" should be recognised as separated species. Moreover, as emphasised by Teixeira et al. (2022), ecological factors and selective pressures mediated by the pollinators should be the drivers of the speciation process in *Nicotiana* that occurred along the escarpments of the oriental border in southern Brazilian plateau.

Finally, we highlight the importance of using distinct biological information sources and techniques to accurately determine taxa boundaries and make more confident taxonomic decisions, what is crucial for describing diversity (Dayrat, 2005). This is particularly important in the study of species complexes in the Neotropical region, where few species have been examined under this perspective (Pinheiro, Dantas-Queiroz, & Palma-Silva, 2018; Souza, Andrade, & Stehmann, unpublished data), and the boundaries of many of them are not easily recognisable.

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SUPPORTING INFORMATION

Table S1. Sample information for *N. forgetiana* and “N. rastroensis”.

Taxon	**Pop**	**Geographic coordinates**	**Collector number (*Voucher*)**	**N**	**Haplotypes**
N. forgetiana morphotype rastroensis	Ras 01*	28°23'59.7"S 49°33'03.7"W	Turchetto, C. 02 (ICN201470)	5	Hap_8
	Ras 02*	28°04'02.9"S 49°21'23.2"W	Turchetto, C. 08 (ICN201475)	4	Hap_9
	Ras 03	28°48'35"S 49°55'45.32"W	Augsten, M. 1138 (BHCB 192536)	3	Hap_10
N. forgetiana	Forg 01*	28°40'14.8"S 51°03'32.3"W	Turchetto, C. 18 (ICN201486)	4	Hap_1
	Forg 02*	29°00'00.7"S 51°04'16.4"W	Turchetto, C. 21 (ICN201489)	6	Hap_2
	Forg 03*	28°33'05.40"S 52°36'40.0"W	Turchetto, C. 27 (ICN201495)	7	Hap_2
	Forg 04*	28°30'19.0"S 51°42'02.7"W	Augsten, M. 1160 (BHCB 202363)	8	Hap_3
	Forg 05*	29°08'42.0"S 51°32'01.7"W	Augsten, M. 1177 (BHCB 202380)	7	Hap_1
	Forg 06	29°16'16.72"S 50°44'28.32"W	Augsten, M. 1144 (BHCB 192542)	8	Hap_2, Hap_4, Hap_5, Hap_6
	Forg 07	29°6'21.794"S 51°3'46.123"W	Augsten, M. 1145 (BHCB 192543)	4	Hap_2, Hap_7

Pop – collection site code; N – sampling size; * samples included in morphometric analyses (Teixeira *et al.* 2022). ICN – Universidade Federal do Rio Grande do Sul herbarium, Porto Alegre, Brazil; BHCB - Universidade Federal de Minas Gerais herbarium, Belo Horizonte, Brazil

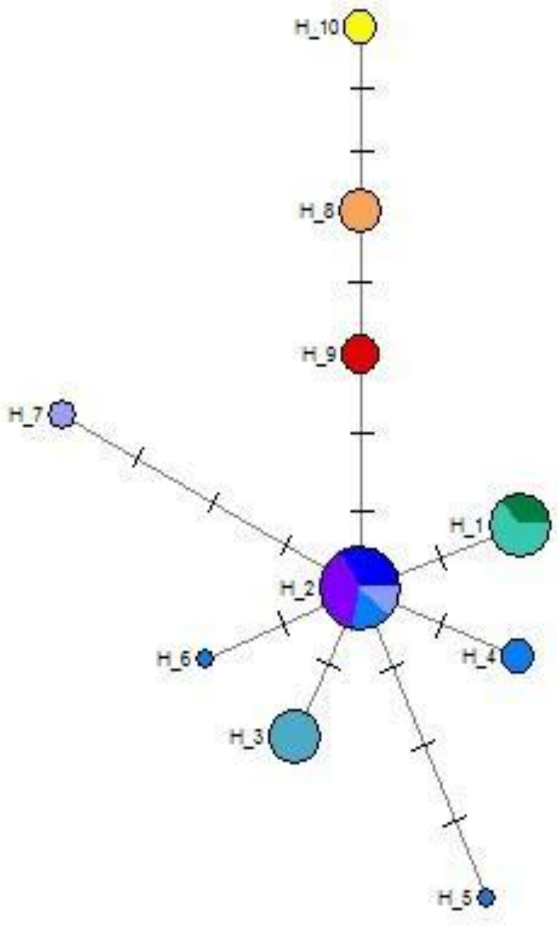


Figure S1. The haplotype relationships obtained using median joining network algorithm. Circle diameter is proportional to haplotype frequency. Each colour indicates population. Perpendicular lines correspond to one mutational step. H_1 to H_7 were found in *N. forgetiana* individuals*,* and H_8 to H_10 in *N. forgetiana* morphotype rastroensis,.

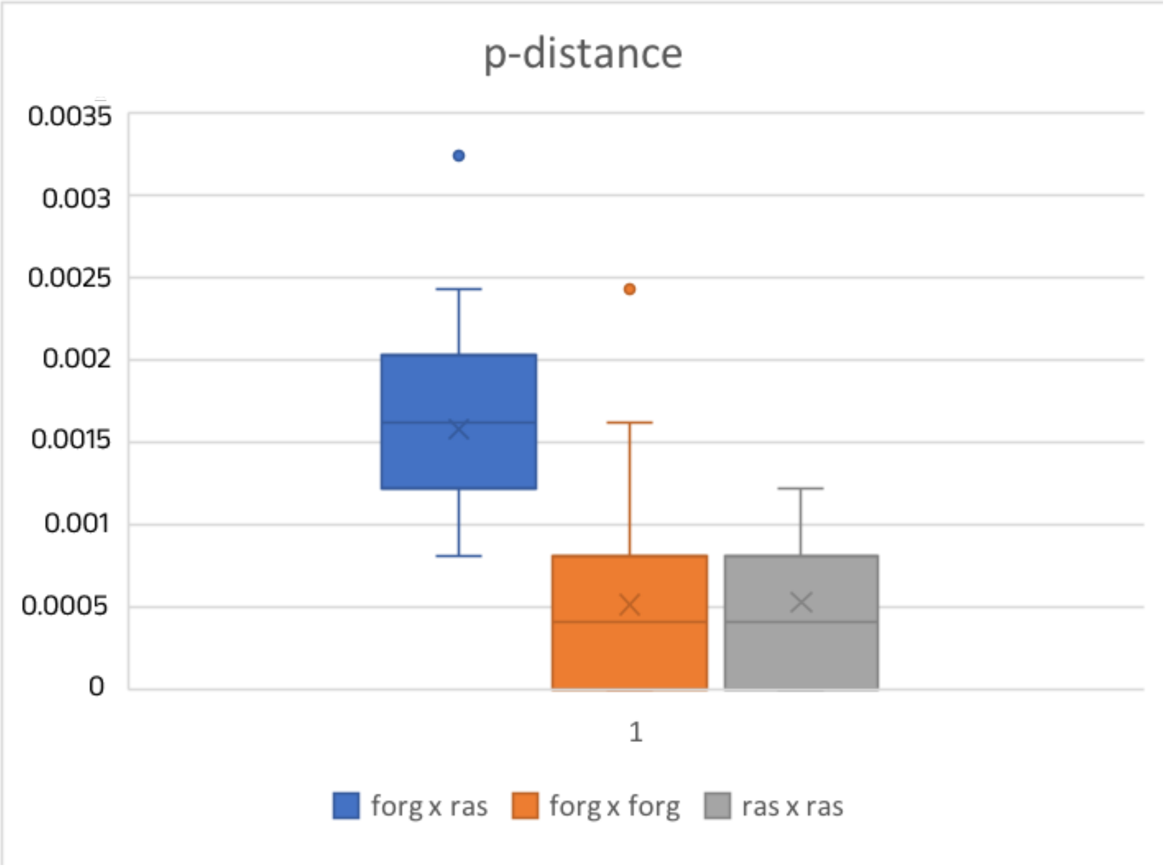


Figure S2: Intraspecific and interspecific p-distances among *N. forgetiana* morphotype rastroensis and *N. forgetiana* individuals based on plastid haplotypes.

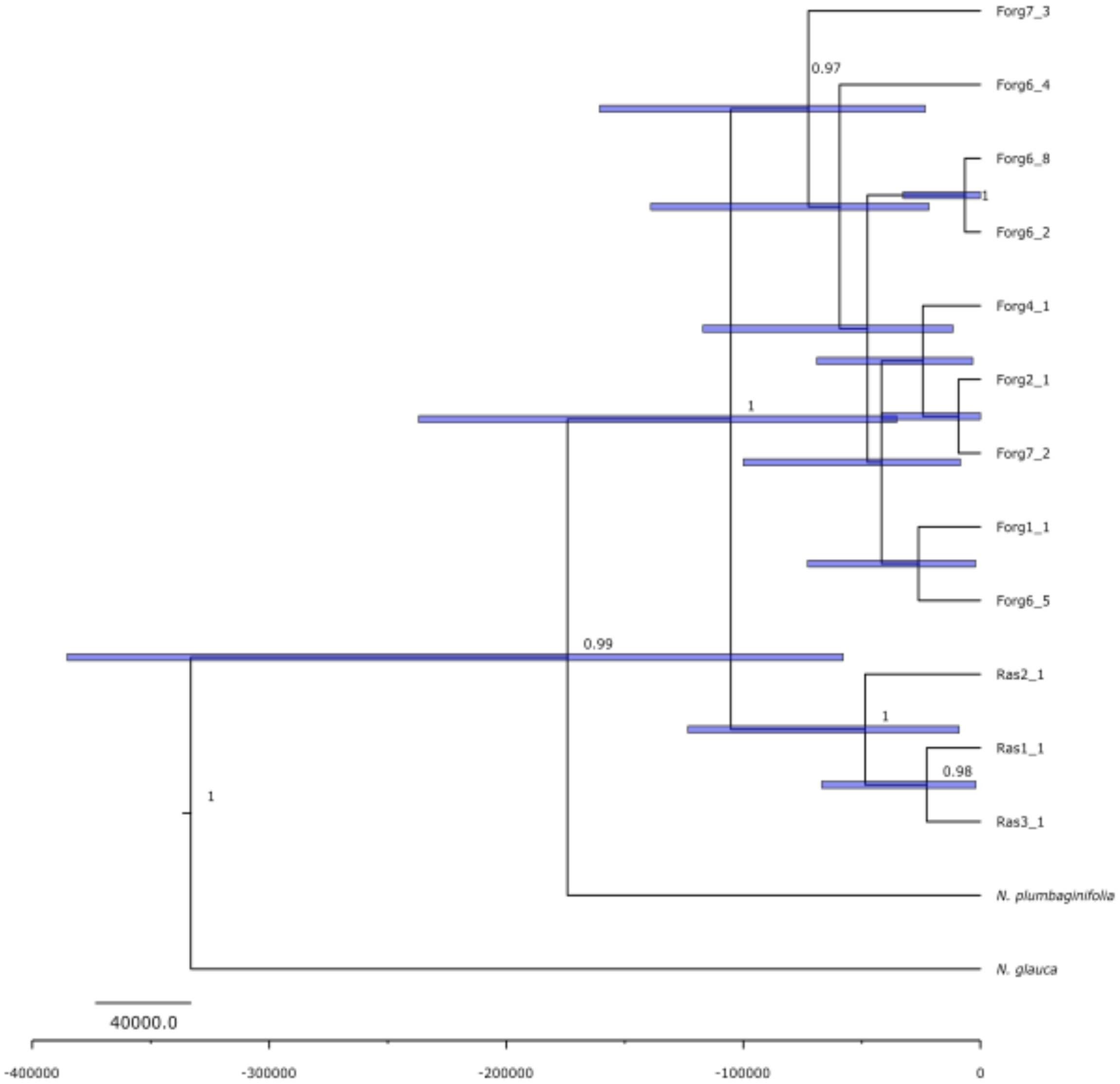


Figure S3. Dated phylogenetic tree

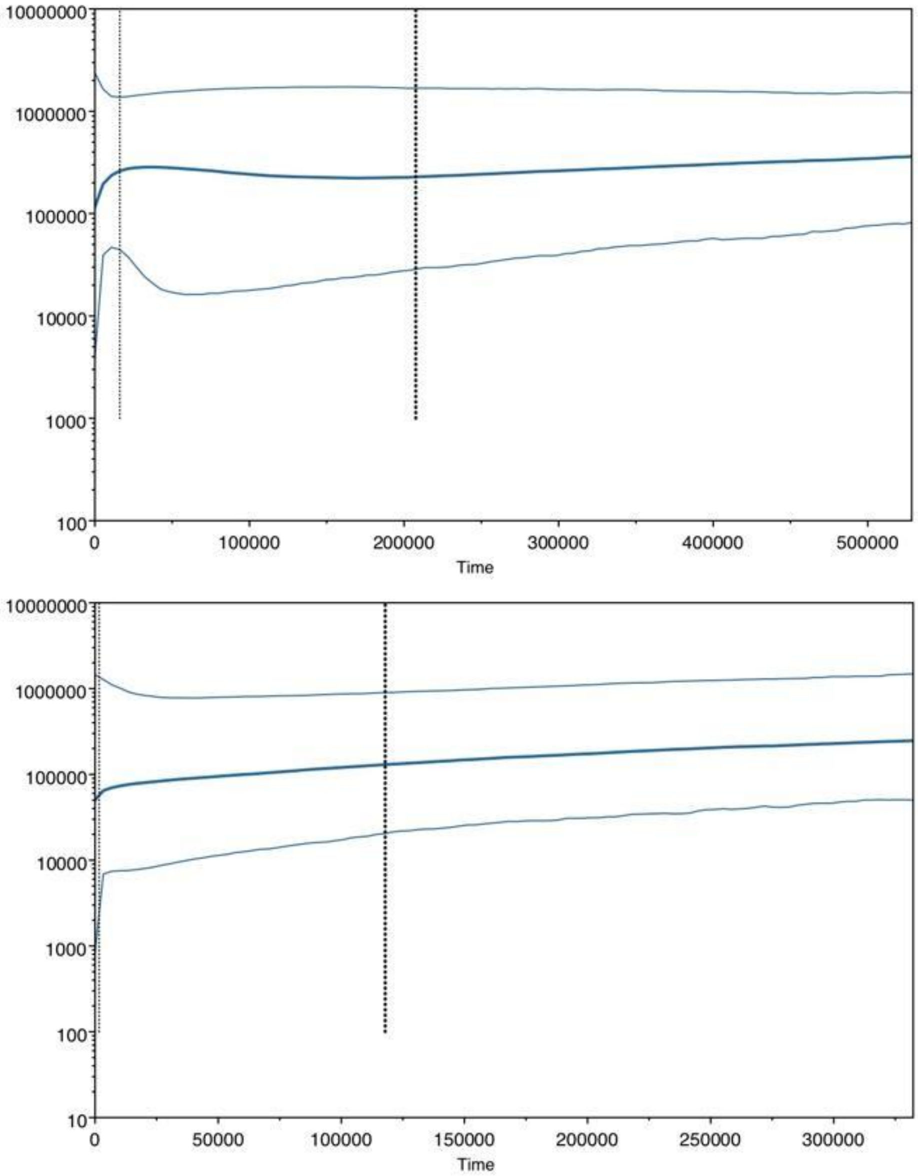
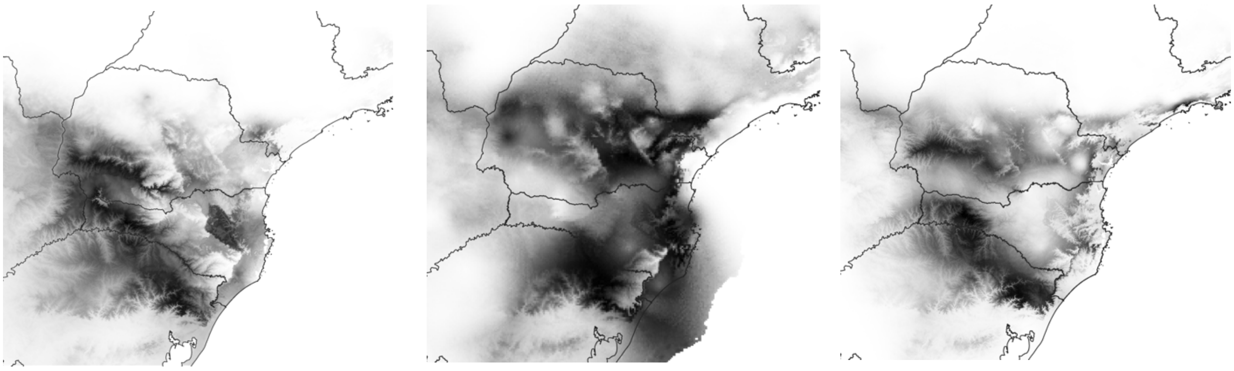


Figure S4: Demographical changes over time estimated using Bayesian skyline plot in Beast for *N. forgetiana* (A) and *N. forgetiana* morphotype rastroensis (B). Darker line indicates median estimation, lighter lines correspond to confidence intervals.

Present Last Glacial Maximum Last Interglacial

Nicotiana forgetiana



“Nicotiana rastroensis”

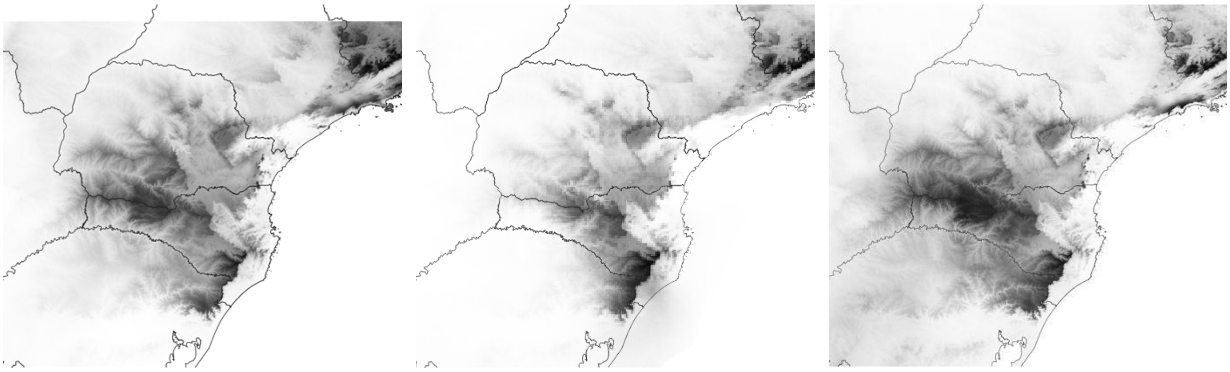


Figure S5: Ecological niche modelling for *Nicotiana forgetiana* and *N. forgetiana* morphotype rastroensis considering three periods: current distribution (1970–2000), Last Glacial Maximum (LGM; *ca.* 21 kya), and Last Interglacial (LIG; *ca.* 120–140 kya). Darker areas correspond to higher suitability.

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CONSIDERAÇÕES FINAIS

Neste trabalho buscou-se compreender melhor a diversidade de *Nicotiana* sect. *Alatae* (Solanaceae), grupo que ocorre majoritariamente no Brasil. Os principais resultados foram: a descrição de uma espécie nova, *N. gandarela* Augsten & Stehmann*,* e o estudo de uma espécie críptica em *N. forgetiana* Hemsl., utilizando a taxonomia integrativa. A descrição de *Nicotiana gandarela* contou com a inclusão da espécie na filogenia do gênero, encontrando como resultado a confirmação de que a espécie pertence à seção *Alatae*, e além disso está posicionada como grupo irmão de todas as espécies da seção. No caso do complexo envolvendo *N. forgetiana s.l.*, a partir de regiões de cloroplasto e modelagem de nicho ecológico, somado as evidências morfológicas, verificou-se a ocorrência de uma espécie críptica, a qual chamamos *N. forgetiana* morfotipo rastroensis As populações desta espécie ocorrem nos cânions da Serra Geral entre Rio Grande do Sul e Santa Catarina, compõe uma espécie distinta daquela que ocorre no interior do Rio Grande do Sul. A descrição da nova espécie está em preparação, mas depende da análise detalhada do material tipo e documentos históricos de sua coleta, que se encontram, respectivamente, no herbário e nos arquivos da biblioteca do Royal Botanic Gardens, Kew.

Apesar do avanços, há muitas lacunas de conhecimento em *N.* sect*. Alatae*, que precisam ser preenchidos. Acreditamos que este grupo é um excelente modelo para estudos evolutivos, por possuir esta diversidade de diferentes síndromes de polinização, e por se tratar de um gênero com relevância científica destacada.