UNIVERSIDADE DE BRASÍLIA
DEPARTAMENTO DE FITOPATOLOGIA

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Informatividade filogenética de marcadores para identificação de espécies de *Septoria*: Estudo de caso em Asteraceae e Ericaceae

Brasília, DF
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Dissertação apresentada ao Programa de Pós-Graduação em Fitopatologia da Universidade de Brasília como parte dos requisitos para a obtenção do título de Mestre em Fitopatologia.
Orientador: Prof. Danilo Batista Pinho.

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Aprovado em 28 de fevereiro de 2025.

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Informatividade filogenética de marcadores para identificação de espécies de *Septoria*: Estudo de caso em Asteraceae e Ericaceae

Resumo

Septoria é um fungo fitopatogênico que causa manchas foliares em diversas espécies de plantas. Inicialmente, a identificação era baseada em características morfológicas e na especificidade do hospedeiro. No entanto, análises filogenéticas revelaram a presença de espécies crípticas e uma menor especificidade quanto ao hospedeiro. Embora sete regiões genômicas sejam amplamente utilizadas para identificação precisa, não há consenso sobre sua real necessidade, e a falta de padronização entre estudos dificulta a comparação entre isolados, aumentando custos e exigindo mais amostras para análise. Este estudo teve como objetivo identificar novas espécies e hospedeiros de *Septoria*, além de avaliar o sinal filogenético de sete regiões genômicas para a delimitação de espécies. Os isolados foram obtidos de manchas foliares de cinco espécies de plantas (*Acanthospermum hispidum, Conyza canadensis, Crepis japonica, Rhododendron simsii* e *Vernonia polysphaera*) coletadas nos estados do Ceará, Goiás, Pernambuco, Paraná, Rio de Janeiro, Santa Catarina, São Paulo e no Distrito Federal. As regiões genômicas actina (*act*), calmodulina (*cmda*), β-tubulina (*tub*2), espaçador interno transcrito do RNA ribossômico (ITS), fator de elongação 1-alpha (*tef*1), 28S nrDNA (LSU) e subunidade maior da RNA polymerase II (*rpb*2) foram sequenciadas para análises multilocus de Máxima Verossimilhança e Inferência Bayesiana, a identificação de espécies foi feita seguindo o critério do reconhecimento de espécies filogenéticas por concordância genealógica (*GCPSR*). A avaliação da informatividade genética das sete regiões genômicas foi feita utilizando uma abordagem integrada utilizando o *PhyDesign* e valores de *Barcode gap distance* e *Inter/intra overlap distance*. O "Fator de Concordância" foi utilizado para a verificar proporção de regiões genômicas que apoiam consistentemente um clado na comparação entre árvores utilizando todos os genes e a árvore utilizando apenas os genes mais informativos. Entre os 27 isolados obtidos de plantas pertencentes as famílias Asteraceae e Ericaceae, apenas *S. crepidis* foi encontrada na mesma hospedeira previamente relatada no Brasil. A espécie *S. siegesbeckiae* foi relatada pela primeira vez em *Acanthospermum hispidum* (Asteraceae). Adicionalmente, este estudo propõe uma nova espécie associada com *Conyza canadensis* e a caracterização molecular de *S.* cf. *vernoniae* associada com *Vernonia polysphaera*. A análise de informatividade filogenética indicou que as regiões genômicas *tef1*, *cmda* e *tub2* são as mais eficazes para a identificação de espécies dentro do gênero *Septoria.* Além disso, se observa um aumento na concordância dos clados bem suportados quando apenas os genes mais informativos são utilizados. Portanto, o sequenciamento dessas regiões é suficiente para a identificação acurada de espécies, enquanto os demais marcadores devem ser empregados na descrição de novas espécies. Esses resultados reforçam a importância da integração de abordagens morfológicas e moleculares para uma classificação mais precisa e padronizada do gênero.

Palavras-chave: Doenças de plantas, gama de hospedeiras, Mycosphaerellaceae, novo táxon, taxonomia.

Phylogenetic informativeness of markers for Septoria species identification: Asteraceae and Ericaceae as a case study

Abstract

Septoria is a phytopathogenic fungus that causes leaf spots in a variety of plant species. Initially, identification was based on morphological characteristics and host specificity. However, phylogenetic analyses have revealed the existence of cryptic species and a reduced host specificity. Although seven genomic regions are widely utilized for accurate species identification, there is no consensus regarding their actual necessity, and the lack of standardization between studies complicates the comparison of isolates, increasing costs and requiring additional samples for analysis. This study aimed to identify new species and hosts of *Septoria* and to evaluate the phylogenetic signal of seven genomic regions for species delimitation. Isolates were obtained from leaf spots of five plant species (*Acanthospermum hispidum, Conyza canadensis, Crepis japonica, Rhododendron simsii,* and *Vernonia polysphaera*) collected from the states of Ceará, Goiás, Pernambuco, Paraná, Rio de Janeiro, Santa Catarina, São Paulo, and the Federal District. The genomic regions actin (*act*), calmodulin (*cmda*), β-tubulin (*tub*2), nuclear ribosomal internal transcribed spacer (ITS), elongation factor 1-alpha (*tef*1), 28S nrDNA (LSU), and the largest subunit of RNA polymerase II (*rpb*2) were sequenced for multilocus Maximum Likelihood and Bayesian Inference analyses. Species identification followed the criterion of phylogenetic species recognition through genealogical concordance (GCPSR). Genetic informativeness of the seven genomic regions was assessed using an integrated approach, including Barcode gap distance, Inter/intra overlap distance and PhyDesign, while the "Concordance Factor" was used to determine the proportion of genomic regions consistently supporting a clade in comparisons between trees constructed with all genes versus those using only the most informative genes. Among 27 isolates obtained from plants belonging to the Asteraceae and Ericaceae families, only *S. crepidis* was found on the same host previously reported in Brazil. The species *S. siegesbeckiae* was first recorded on *Acanthospermum hispidum* (Asteraceae). Additionally, this study proposes a new species associated with *Conyza canadensis* and provides the molecular characterization of *S. cf. vernoniae* associated with *Vernonia polysphaera*. Phylogenetic informativeness analysis showed that the genomic regions *tef*1, *cmda*, and *tub*2 are the most effective for species identification within the genus *Septoria,* with an increase in the concordance of well-supported clades when only the most informative genes were used. Therefore, sequencing these regions is sufficient for accurate species identification, while the other markers should mainly be used for the description of new species. These results emphasize the importance of integrating both morphological and molecular approaches for a more accurate and standardized classification of the genus.

Keywords: Plant diseases, host range, Mycosphaerellaceae, new taxon, taxonomy.

INTRODUCTION

Septoria Sacc. (Dothideomycetes: Ascomycota) is a genus of phytopathogenic fungi commonly associated with leaf spots but also capable of infecting stems, seeds, fruits, and floral organs of plants (Crous *et al*., 2001; 2009; Sousa *et al*., 2003). This genus impacts several crops of global importance, such as blueberry, hemp, lettuce, pistachios, soybeans and tomatoes, leading to substantial economic losses (Sousa *et al*., 2003; Scherm *et al*., 2008; Cruz *et al.,* 2010; Crous *et al*., 2013; Costa *et al*., 2019; Rahnama *et al*., 2021; Neves *et al*., 2022; Costa *et al*., 2024). Lesions are typically circular but may become angular due to spot coalescence or leaf morphology (Sousa *et al*., 2003; Costa *et al*., 2019; Costa *et al*., 2024). Small brownish spots on the leaves evolve into dark spots with defined edges, surrounded by chlorotic halos that range from yellowish to bronze (Sousa *et al*., 2003; Cruz *et al*., 2010; Rahnama *et al*., 2021; Costa *et al*., 2019; Costa *et al*. 2024).

The genus *Septoria* was proposed as a pathogen of *Cytisus laburnum* (= *Laburnum anagyroides*), with *S. cytisi* designated as the type species (Desmazières, 1847; Sutton, 1980; Quaedvlieg *et al*., 2013). Over time, more than 2,000 species have been assigned to *Septoria*, most lacking a known sexual form (Desmazières, 1847; Sutton, 1980; Farr, 1991, 1992, Verkley & Willemse, 2004).

The genus is recognized as one of 23 asexual morphs of *Mycosphaerella* (Crous *et al.*, 2001; 2009; Sousa *et al*., 2003). But, unlike most *Mycosphaerella* asexual forms, which produce fasciculate conidiophores (e.g., *Cercospora, Ramularia,* and *Pseudocercospora*), *Septoria* is characterized by pycnidia containing hyaline, filiform phragmospores, with conidiophores reduced to determinate or indeterminate conidiogenous cells (Sutton, 1980; Crous *et al*., 2001; Verkley *et al*., 2004; Quaedvlieg *et al*., 2013; Verkley *et al*., 2013; Bakhshi *et al*., 2019). Under the one fungus, one name system, the epithet *Mycosphaerella* has been assigned exclusively to the sexual form of the genus *Ramularia*, whereas *Septoria* remains an independent lineage (Aptroot, 2006; Quaedvlieg *et al*., 2011).

Historically, the delimitation of *Septoria* species were based on morphological characteristics and host specificity (Desmazières, 1847; Sutton, 1980; Farr, 1991, 1992; Verkley & Willemse, 2004). The integration of molecular data, revealing homoplasy into *Septoria*-like fungi, allowed the reclassification of several species into

other genera, such as *Zymoseptoria* and *Parastagonospora* (Quaedvlieg *et al*., 2011, 2013). Furthermore, it was proved that a species can infect hosts from different botanical families, showing that there may be an overestimation of the number of species (Verkley *et al*., 2013; An *et al*., 2021).

The molecular characterization of approximately 115 *Septoria* species has contributed to the taxonomy of the genus; however, the holotype is unavailable for several of the known species (Verkley & Willemse, 2004; Verkley *et al*., 2013; Quaedvlieg *et al*., 2013). The phylogeny of *Septoria* has been conducted through multigene analyses based on partial sequencing of genomic regions of DNA (Feau *et al*., 2006; Verkley *et al*., 2013; Zalewska *et al*., 2017; Bakhshi *et al*., 2019; An *et al*., 2021).

To date, there is no consensus on the number or selection of the most appropriate gene regions for species delimitation (Verkley *et al*., 2013; Costa *et al*., 2021; Cabral *et al*., 2021). While some studies use a few gene regions (Verkley *et al*., 2004; Erper & Tunali, 2010, Newbery *et al*., 2020, Gusella *et al*., 2021), others use five or more (Verkley *et al*., 2013; Bakhshi *et al*., 2019; An *et al*., 2021). Furthermore, the use of different gene combinations limits their application in comparative analyses across studies (Feau *et al*., 2006; Verkley *et al*., 2013).

The informativeness of actin (*act*), calmodulin (*cmda*), β-tubulin (*tub*2), nuclear ribosomal internal transcribed spacer (ITS), elongation factor 1-alpha (*tef*1), 28S nrDNA (LSU), and the largest subunit of RNA polymerase II (*rpb*2) genes were previously evaluated (Verkley *et al.*, 2013), however, certain genic regions remain underutilized (Feau *et al*., 2006). Thus, it is crucial to assess not only the number of genes but also their effectiveness and their contribution to a more robust phylogeny of the genus *Septoria*. In Brazil, 51 *Septoria* species have been associated with 78 hosts (Fungal Database, 2025). However, only *S. lycopersici* and *S. lactucae* have been molecularly characterized (Costa *et al*., 2021; Cabral *et al*., 2021).

Therefore, the objectives of this study are to: (*i*) Identify *Septoria* species associated with different hosts in the Asteraceae and Ericaceae families and (*ii*) evaluate the performance of commonly used genomic regions for species identification.

2. MATERIALS AND METHODS

2.1. Sampling and Isolation

Symptomatic leaves were collected from different host plants in the states of Ceará, Pernambuco, Paraná, Rio de Janeiro, Santa Catarina, São Paulo, and the Federal District (Suplementary Table 1), between August 2021 and January 2024. The samples were kept in a humid chamber for 24 to 48 hours to promote spore production, Subsequently, single-spore cultures were established on potato dextrose agar (PDA) medium, at 25ºC for 14 days. Then, the isolates were stored in the *Coleção de Culturas da Universidade de Brasília* (CCUB).

2.2. DNA extraction, PCR and sequencing

The *Septoria* isolates were grown in Petri dishes containing PDA medium, at 25 ºC for 7-21 days. The mycelial growth was collected with a sterile toothpick and deposited in 1.5mL microtubes containing 40µL of Tris-EDTA buffer (TE) and four metal beads (2.8 mm). Total DNA extraction was performed using the *Wizard Genomic DNA Purification* kit from Promega® according to the manufacturer's instructions. The total DNA was analyzed by electrophoresis in 1% agarose gel, stained with GelRed (Biotium®) and visualized under ultraviolet light. The DNA samples were stored at -20 ºC.

The isolates were sequenced for seven loci: actin (*act*), calmodulin (*cmda*), β-tubulin (*tub*2), nuclear ribosomal internal transcribed spacer (ITS), elongation factor 1-alpha (*tef*1), 28S nrDNA (LSU), and the largest subunit of RNA polymerase II (*rpb*2). The PCR amplifications were performed in a final volume of 12.5 µL: 6.25 µL of MyTaq MasterMix 2x (Bioline®, EUA), 0.3 µL (10 pmol/mL) of each primer, 4.25 µL of nuclease-free water, and 1 ml of template DNA. The cycling conditions were initial denaturation at 95 ºC for 1.5 min, followed by 35 cycles at 95 ºC for 20 s, 45s for annealing (the temperatures for each set of primers are listed in **Table 1**), 72 ºC for 1 min for extension, and a final extension at 72 ºC for 5 min. The amplicons were purified and bidirectionally Sanger sequenced.

The new sequences were assembled and manually edited using DNADragon v.1.5.1 (SequentiX – Digital DNA Processing, Klein Raden, Germany). To confirm whether the sequenced isolates belonged to the genus *Septoria*, the BLASTn

algorithm (Altschul et al., 1990) was used and the sequences were deposited in GenBank.

2.4. Sequence alignment and phylogenetic analyses

Sequences of ex-type and reference isolates of *Septoria* from previous studies were retrieved from GenBank and included in the analyses (Feau *et al*., 2006; Crous *et al*., 2013; Quaedvlieg *et al*., 2013; Verkley *et al*., 2013; Zalewska *et al*., 2017; Bakhshi *et al*., 2019; An *et al*., 2021; Cabral *et al*., 2021; Costa *et al*., 2021; Ujata *et al*., 2024).

The sequences were compiled using the GenBank tool implemented in MEGA v.7 (Kumar *et al*., 2016). A basic alignment of the obtained sequence data was performed using MAFFT v.7 (https://mafft.cbrc.jp/alignment/server/index.html; Katoh *et al*., 2019; Kuraku *et al*., 2013) and manually edited when necessary using MEGA v.7.

The phylogeny for each locus and the concatenated matrices were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. The multiple sequence alignments were partitioned into multilocus matrices using SequenceMatrix 1.8 (Vaidya *et al*., 2011). ML analyses were performed using IQ-TREE v. 2.3.6 (Nguyen *et al*., 2015). Model parameters were estimated for each partition using ModelFinder (Kalyaanamoorthy *et al*. 2017, Minh *et al*. 2020) allowing each partition to have its evolution rate (Supplementary). The best ML tree was found after 1000 iterations with a perturbation strength of 0.1. Branch supports were estimated using the approximate likelihood-ratio test with Shimodaira–Hasegawa interpretation (SH-alrt) (Shimodaira 2002) with 1000 bootstrap samples. Clades were well supported when SH-alrt bootstrap support was ≥ 80%.

Bayesian inference analyses were conducted using MrBayes 3.2.6 (Ronquist *et al*., 2012), applying the nucleotide substitution models selected based on AICc criteria in MrModeltest 2.3 (Nylander, 2004) (Supplementary Table 2). The process included four parallel runs, each consisting of four MCMC chains, running for 10^7 generations with data sampled every 1,000 generations and posterior probabilities were calculated after excluding the first 25% of sampled generations as burn-in. The trees were visualized using the FigTree v1.4.2 program (Rambaut, 2014).

2.5. Species delimitation

The genealogical concordance phylogenetic species recognition (GCPSR) approach (Dettman *et al*. 2003) was used to determine whether a clade could be considered an independent evolutionary lineage. A clade qualified as if it satisfied at least one of two conditions: genealogical concordance or genealogical non-discordance. Genealogical concordance was achieved if the clade appeared in the majority of individual gene trees. Genealogical non-discordance required the clade to have strong support in at least one gene tree, with ML (≥80%) and BI (≥0.95) analyses, and no contradictions in other gene trees at the same support level. Novel species were identified when a clade met the criteria for independent evolutionary lineages, showed robust support in both ML and BI analyses of the 7-locus concatenated dataset, and was not nested within clades containing the type specimens of previously described species in either analysis.

Table 1. Primers used for the fungal loci amplification, including their sequences, annealing temperatures, and orientations

Locus	Primer	Primer sequence 5' to 3'	Annealing temperature (°C)	Orientation	Reference
act	512F	ATGTGCAAGGCCGGTTTCGC	60	Foward	Carbone & Kohn, 1999
	783R	TACGAGTCCTTCTGGCCC AT		Reverse	
cmda	228F	GAGTTCAAGGAGGCCTTCTCCC	55	Foward	Carbone & Kohn, 1999
	2RD	TGRTCNGCCTCDCGGATCATCTC		Reverse	Groenewald *et al*., 2013
ITS	V9G	TTACGTCCCTGCCCTTTGTA	53	Foward	Hoog & Gerrits van den Ende, 1998
	LR5	TCCTGAGGGAAACTTCG		Reverse	Vilgalys & Hester, 1990
LSU	V9G	TTACGTCCCTGCCCTTTGTA	53	Foward	Hoog & Gerrits van den Ende, 1998
	LR5	TCCTGAGGGAAACTTCG		Reverse	Vilgalys & Hester, 1990
rpb2	5F2	GGGGWGAYCAGAAGAAGGC	54	Foward	Sung *et al*., 2007
	7cR	CCCATRGCTTGYTTRCCCAT		Reverse	Liu *et al*., 1999
tef1	EF1F	TGCGGTGGTATCGACAAGCGT	56	Foward	Jacobs *et al*., 2004
	EF2R	AGCATGTTGTCGCCGTTGAAG		Reverse	
tub2	T1	AACATGCGTGAGATTGTAAGT	53	Foward	O'Donnell & Cigelnik, 1997
	T22	TCTGGATGTTGTTGGGAATCC		Reverse	

2.6. Optimal genomic regions for species identification of Septoria

To evaluate the performance of the best genomic regions, two approaches were applied: the *barcode gap distance* and *intra/inter-overlap distance* method (Hebert *et al*., 2003), as well as the PhyDesign (López-Giráldez & Townsend, 2011), following the workflow of Vieira *et al*., 2020. According to Hebert *et al*., 2003 the *barcode gap*

distance is the difference between the average genetic distances between species (interspecific) and within a species (intraspecific). A large *barcode gap* makes it easier to distinguish between species. The *intra/inter-overlap distance* measures the overlap between intraspecific and interspecific genetic distances. A high overlap indicates that the sequenced region is ineffective, as the distances between and within species are too similar. Ideally, this overlap should be minimal for the sequenced region to be effective.

Barcode gap distance and *intra/inter-specific overlap distance* were calculated for single-locus alignments in MEGA 7, excluding single-isolate species. The Kimura-2-parameter model was used, accounting for differences in transition and transversion rates, uniform site rates, and treating gaps as pairwise deletions. Distances were organized in Microsoft Excel, and maximum, minimum, and mean values were calculated. The *barcode gap* was defined as the difference between mean interspecific and intraspecific distances, while overlap percentage was calculated as max intraspecific distance ÷ max interspecific distance × 100. Effective barcodes have a large *barcode gap* and low *overlap*.

PhyDesign evaluates site rate estimates and phylogenetic informativeness, prioritizing them according to the epochs of interest (López-Giráldez & Townsend, 2011). To obtain site rate distributions for each locus, two datasets are required: (1) an alignment of the loci of interest, pruned to contain a set of taxa for which the tree topology is relatively well known, and (2) an ultrametric tree for those taxa (López-Giráldez & Townsend, 2011). For this step, a Maximum Likelihood tree was constructed in IQ-TREE v. 2.3.6 (Nguyen *et al*. 2015) using one isolate of each species that contained all seven regions. The ML tree was then converted into a rooted ultrametric tree using the 'chronos' function in the *ape* package (Paradis *et al*., 2004) in R Studio 1.1.442 (R Core Team, 2017). The trees were calibrated using an arbitrary timescale, with time = 0 at the tips and time = 1 at the root. The ultrametric trees and the corresponding partitioned alignment were used as input files in PHYDESIGN, and substitution rates were calculated using the HyPhy program (Pond *et al*., 2005). The substitution rates estimated by HyPhy's maximum likelihood algorithm were unrealistic for some sites in the alignment, resulting in biologically implausible "ghost" peaks at very recent time points. These peaks are likely due to indels or ambiguous sites in the

alignment; therefore, alignment positions with poorly estimated substitution rates were excluded from some genes before generating the phylogenetic informativeness profile, as recommended by the authors of PhyDesign (López-Giráldez & Townsend, 2011). The phylogenetic informativeness values (PIV) were calculated on a net basis. The variable Pimax represents the time at which a given marker reaches its maximum PIV and was used to determine the divergence time at which the marker is most informative (Fong and Fujita, 2011). Markers were ranked according to their PIV values, and their utility was assessed based on the profile shape: low and flat curves indicate the least informative markers, while high and sharp peaks correspond to the most informative ones.

The selection of a minimal set of optimal markers for phylogenetic inference within the genus was carried out based on phylogenetic informativeness profiling (Vieira *et al*., 2020). The markers were ranked and selected according to their PIV values. Subsequently, maximum likelihood (ML) trees were generated from the concatenation of the three most informative markers. If any species clade showed low statistical support or not all species were consistently recovered compared to the multilocus analyses that included all markers (unresolved relationships/polytomy), additional markers would be progressively concatenated in descending order of their phylogenetic informativeness until all species were adequately resolved.

The efficiency of using seven and three genomic regions was assessed through the comparison of support values and concordance factors (Farris *et al.,* 1995). The aim was to determine whether removing the least effective markers would enhance support and improve the proportion of genes that resolve species, thereby better aligning with the GCPSR criteria.

To quantify the proportion of markers supporting a given clade, the Concordance Factor (CF) was calculated in Maximum Likelihood (ML) analyses for each combination of genomic regions. A CF value above 50% indicates that the clade is well-resolved in the phylogenetic tree (Müller, 2004). Phylogenetic analyses and CF calculations were performed using the IQ-TREE2 v. 2.3.6 software (Nguyen *et al*. 2015), considering only species with all seven gene regions available. Species with missing data were excluded to ensure the reliability of the results.

3. RESULTS

3.1 *Phylogenetic analyses*

Multilocus Maximum Likelihood (ML) and Bayesian Inference (BI) analyses identify four different species (Figure 1). Fourteen isolates were assigned to *S. crepidis* (CCUB: 3400, 3402, 3405, 3410, 3412, 3416, 3422, 3565, 3566, 3567, 3570, 4577), while four were identified as *S. siegesbeckiae* (CCUB: 3265, 3267, 3270, and 4523). The isolates CCUB 3397, 3398, 3403, 3404, 3413, 3417, and 4546 were molecularly identified as *S.* cf. *vernoniae*, while isolates CCUB 4513 and CCUB 4515 were designated as new species.

All the species mentioned exhibited well-supported branches in the phylogenetic trees generated through both Maximum Likelihood and Bayesian Inference analyses, indicating robust phylogenetic resolution in both methodologies.

Host non-specificity was observed in one of the four species studied. *S. crepidis* were reported on *Crepis japonica* (*Asteraceae*) and *Rhododendron simsii* (*Ericaceae*) (Figure 2). Furthermore, isolates obtained from *Conyza canadensis* were identified as a new species, while isolates obtained from *Vernonia polysphaera* (*Asteraceae*) were classified as *S.* cf. *vernoniae*.

3.2 *Taxonomy*

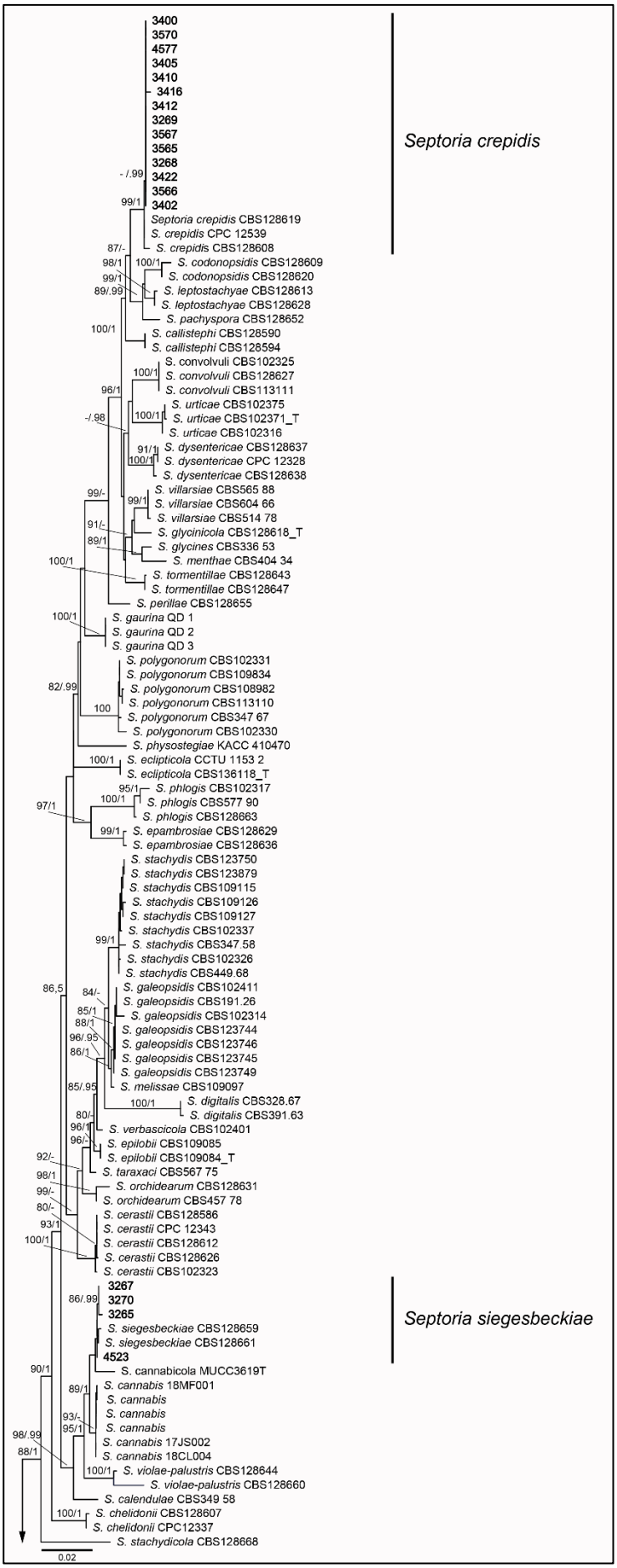
***Septoria* sp. nov 1** Queiroz Júnior, Reis & Pinho. **sp. nov.**

Mycobank: -

Etymology: The name refers to the plant host, from which the fungus was collected. *Typification*: BRAZIL, Rio de Janeiro: Sumidouro, from leaves of *Conyza canadensis* November 2021, coll. A. Reis. (holotype - permanently preserved in a metabolically inactive state, ex-type living culture CCUB 4658).

Teleomorph: Unknown.

Description in vitro: Colonies on Potato Dextrose Agar (PDA) reach 12 to 20 mm in diameter after 2 weeks of growth. The mycelium is dense and exhibits a coloration ranging from gray to black, forming a compact structure. The growth is slightly irregular


Septoria crepidis
Septoria siegesbeckiae
3400
3570
4577
3405
3410
3416
3412
3269
3567
3565
3268
3422
3566
3402
Septoria crepidis CBS128619
S. crepidis CPC 12539
S. crepidis CBS128608
S. codonopsidis CBS128609
S. codonopsidis CBS128620
S. leptostachyae CBS128613
S. leptostachyae CBS128628
S. pachyspora CBS128652
S. callistephi CBS128590
S. callistephi CBS128594
S. convolvuli CBS102325
S. convolvuli CBS128627
S. convolvuli CBS113111
S. urticae CBS102375
S. urticae CBS102371_T
S. urticae CBS102316
S. dysentericae CBS128637
S. dysentericae CPC 12328
S. dysentericae CBS128638
S. villarsiae CBS565 88
S. villarsiae CBS604 66
S. villarsiae CBS514 78
S. glycinicola CBS128618_T
S. glycines CBS336 53
S. menthae CBS404 34
S. tormentillae CBS128643
S. tormentillae CBS128647
S. perillae CBS128655
S. gaurina QD 1
S. gaurina QD 2
S. gaurina QD 3
S. polygonorum CBS102331
S. polygonorum CBS109834
S. polygonorum CBS108982
S. polygonorum CBS113110
S. polygonorum CBS347 67
S. polygonorum CBS102330
S. physostegiae KACC 410470
S. eclipticola CCTU 1153 2
S. eclipticola CBS136118_T
S. phlogis CBS102317
S. phlogis CBS577 90
S. phlogis CBS128663
S. epambrosiae CBS128629
S. epambrosiae CBS128636
S. stachydis CBS123750
S. stachydis CBS123879
S. stachydis CBS109115
S. stachydis CBS109126
S. stachydis CBS109127
S. stachydis CBS102337
S. stachydis CBS347.58
S. stachydis CBS102326
S. stachydis CBS449.68
S. galeopsidis CBS102411
S. galeopsidis CBS191.26
S. galeopsidis CBS102314
S. galeopsidis CBS123744
S. galeopsidis CBS123746
S. galeopsidis CBS123745
S. galeopsidis CBS123749
S. melissae CBS109097
S. digitalis CBS328.67
S. digitalis CBS391.63
S. verbascicola CBS102401
S. epilobii CBS109085
S. epilobii CBS109084_T
S. taraxaci CBS567 75
S. orchidearum CBS128631
S. orchidearum CBS457 78
S. cerastii CBS128586
S. cerastii CPC 12343
S. cerastii CBS128612
S. cerastii CBS128626
S. cerastii CBS102323
3267
3270
3265
S. siegesbeckiae CBS128659
S. siegesbeckiae CBS128661
4523
S. cannabicola MUCC3619T
S. cannabis 18MF001
S. cannabis
S. cannabis
S. cannabis
S. cannabis 17JS002
S. cannabis 18CL004
S. violae-palustris CBS128644
S. violae-palustris CBS128660
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S. chelidonii CPC12337
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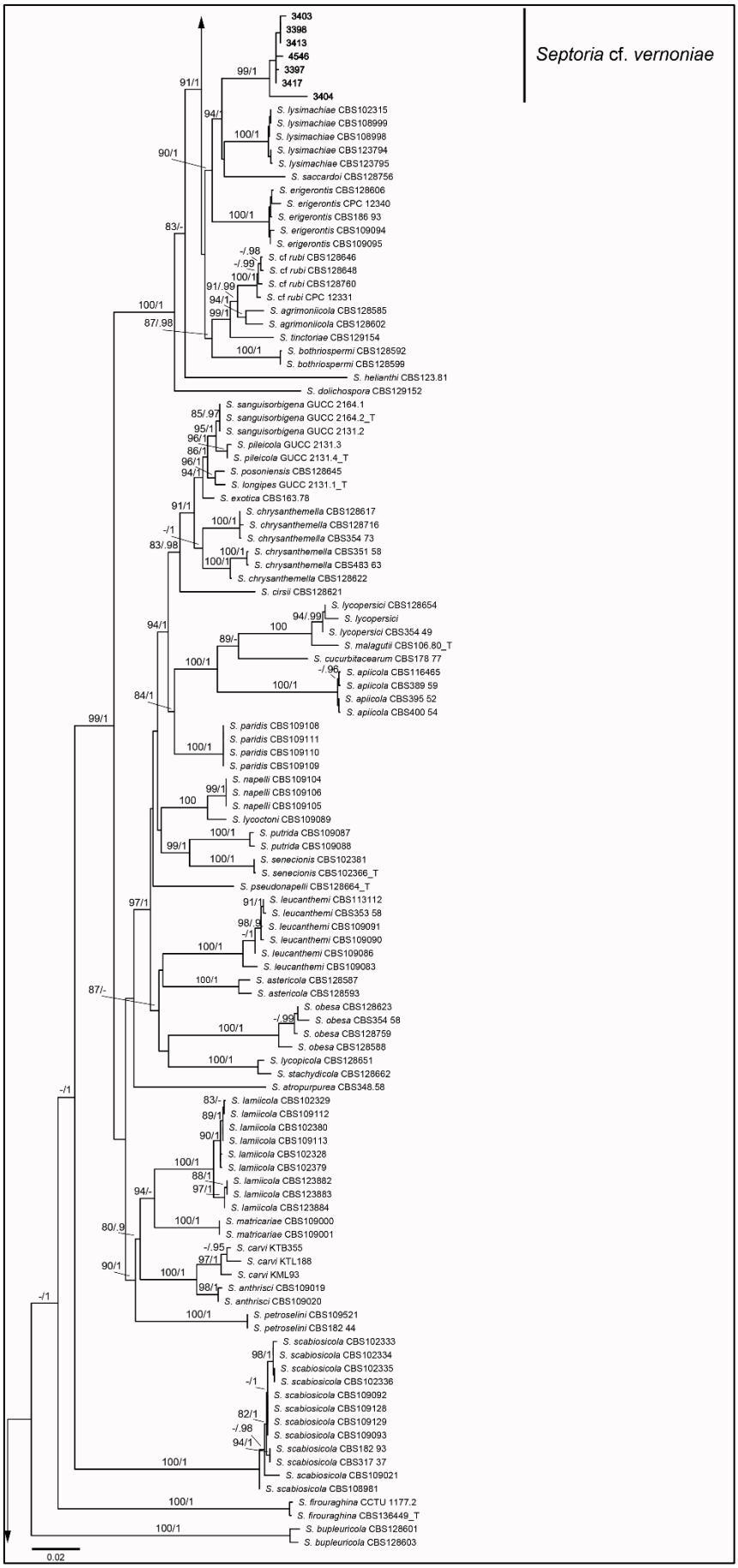
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3397
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S. lysimachiae CBS123794
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S. erigerontis CBS128606
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S. erigerontis CBS186 93
S. erigerontis CBS109094
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S. cf rubi CPC 12331
S. agrimoniicola CBS128585
S. agrimoniicola CBS128602
S. tinctoriae CBS129154
S. bothriospermi CBS128592
S. bothriospermi CBS128599
S. helianthi CBS123.81
S. dolichospora CBS129152
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S. sanguisorbigena GUCC 2164.2_T
S. sanguisorbigena GUCC 2131.2
S. pileicola GUCC 2131.3
S. pileicola GUCC 2131.4_T
S. posoniensis CBS128645
S. longipes GUCC 2131.1_T
S. exotica CBS163.78
S. chrysanthemella CBS128617
S. chrysanthemella CBS128716
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S. chrysanthemella CBS483 63
S. chrysanthemella CBS128622
S. cirsii CBS128621
S. lycopersici CBS128654
S. lycopersici
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S. malagutii CBS106.80_T
S. cucurbitacearum CBS178 77
S. apiicola CBS116465
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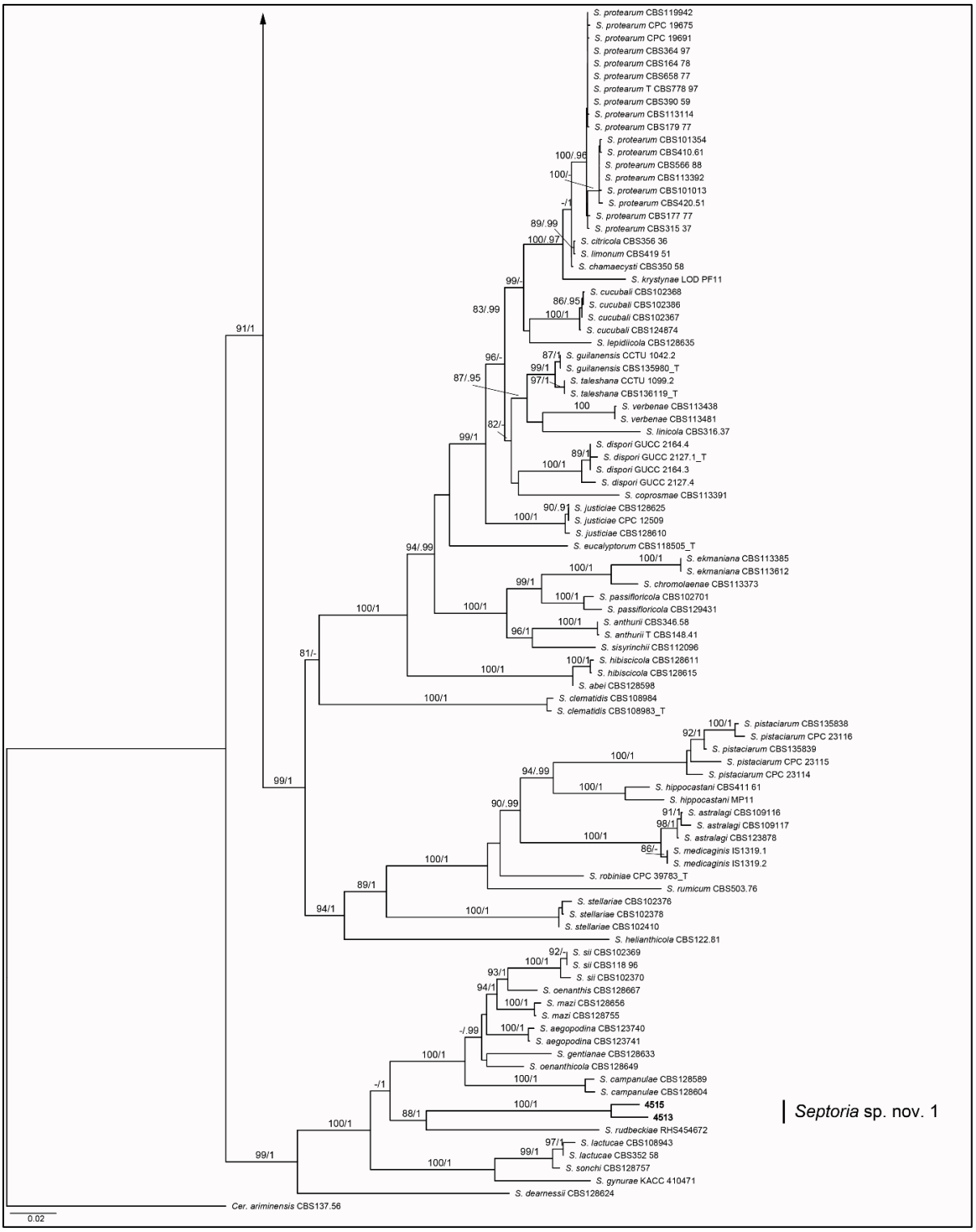

Figure 1. Maximum Likelihood phylogenetic tree based on concatenated sequences (*act*, *cal*, ITS, LSU, *rpb*2, *tef*1 and *tub*2) of *Septoria* species. Bayesian posterior probability and maximum likelihood bootstrap support values are indicated on the branches, the scale bar represents the number of expected changes per site. Significant support (SH-alrt bootstrap support ≥ 80% for ML and posterior probabilities ≥ 0.95 for Bayesian inference) are shown above the nodes. "_T" = type specimen.

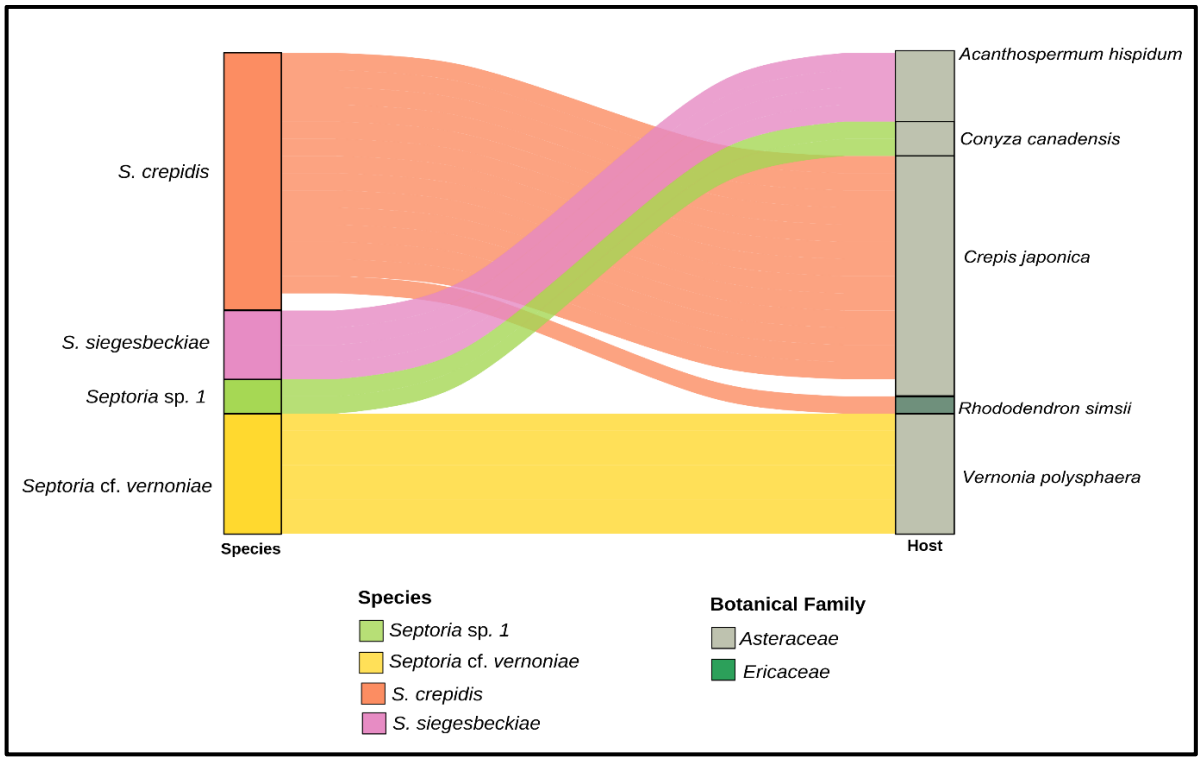

Figure 2. Relationship between *Septoria* species and their hosts. The Sankey diagram illustrates the associations between the identified fungal species and their respective host plants. The colors of the bars represent the different *Septoria* species, while the background of the boxes on the host axis indicates the botanical family.

and radial, with the colonies expanding in a somewhat asymmetrical pattern. The aerial mycelium, visible on the surface of the colony, shows a predominantly gray color, with some areas interspersed in white, suggesting the formation of fungal structures and variations in mycelial development. No reproductive structures were observed.

Additional specimen examined: BRAZIL, Rio de Janeiro: Sumidouro, from leaves of *Conyza canadensis.* November 2021, coll. A. Reis. (CCUB 4657)

Notes: *Septoria* sp. nov. 1 is phylogenetically close to *S. rudbeckiae* but differs by 16, 43, and 25 nucleotides in the ITS, *tef*1 and *tub*2 genes, respectively. The clade corresponding to the species is consistently recovered in a robust manner across all individual gene analyses, with each of the genes showing high branch support, which reinforces the reliability of the phylogenetic relationships observed in each of these sequences.

Figure 3. *Septoria* sp. nov. 1 culture after 7 days on PDA medium.

Septoria protearum Viljoen & Crous, in in Swart, Crous, Denman & Palm, S. Afr. J. Bot. 64(2): 144 (1998).

= *Septoria citricola* Ruggieri, Boll. R. Staz. Patalog. Veget. Roma, N.S. 15: 322 (1935).

= *Septoria chamaecysti* Vestergr. [as 'chamaecisti'] Bih. K. svenska VetenskAkad. Handl., Afd. 3 22(no. 6): 24 (1896).

= *Septoria limonum* Pass., Atti Soc. Crittogam. Ital., Sér. 2 2: 23 (1879).

Notes: The *Septoria protearum* species complex comprises *S. citricola*, *S. chamaecysti*, *S. limonum*, and *S. protearum* (Verkley *et al*. 2013). In the past, *S. citri, S. gerberae, S. hederae,* and *S. lobelia* were synonymized with *S. protearum* due to the minimal base-pair differences in the *rpb2* gene sequences, while the sequences of four other genes were nearly identical (An *et al*., 2021). *S. protearum* is reported as a pathogen in 14 different plant families (Fungal Database, 2025), including Rutaceae, to which *S. limonum* and *S. citricola* were described. On the other hand, *S. chamaecisti* was described in the Cistaceae family, where *S. protearum* has not yet been recorded. However, the differences between *S. citricola, S. chamaecysti* and *S. limonum* in relation to *S. protearum* are limited to variations in the sequences of the actin gene. For the other six analyzed regions, these three species form a single clade grouped with *S. protearum*, with no genetic evidence supporting their distinction as separate species. Furthermore, the inconsistency among phylogenetic trees based on individual

genes and the low statistical support for the branches reinforce the synonymization of *S. citricola, S. chamaecysti* and *S. limonum* with *S. protearum*.

3.3 *Optimal markers for species delimitation of Septoria*

Among the analyzed genes *act*, *cmda, rpb*2, *tef*1 and *tub*2 exhibited high *barcode gap distance* values, surpassing the *overlap* values in the graph (Figure 4). However, despite *tub*2 having the highest *barcode gap distance*, its *overlap* is considerably high, reaching 47.16%. In contrast, *tef*1 and *act* show a high *barcode gap distance* combined with low *overlap*. *Rpb*2 has a *barcode gap distance* is similar to *cmda*, but its *overlap* is relatively high compared to its *barcode gap distance*. Meanwhile, *cmda* presents a *barcode gap* greater than its *overlap*, making it a more informative marker. ITS and LSU exhibited low *Barcode-gap distance* values, indicating their inefficacy for species discrimination. However, despite *tub*2 having the highest *barcode gap distance*, its *overlap* is considerably high, reaching 47.16%. In contrast, *tef*1 and *act* show a high *barcode gap distance* combined with low *overlap*. *Rpb*2 has a *barcode gap distance* like *cmda*, but its *overlap* is relatively high compared to its *barcode gap distance*. Meanwhile, *cmda* presents a *barcode gap* greater than its *overlap*, making it a more informative marker. ITS and LSU exhibited low *Barcode-gap distance* values, indicating their inefficacy for species discrimination.

According to *PhyDesign*, based on *Phylogenetic Informativeness Values* (PIV), the ranking of the markers in descending order is *tef*1 (120), *cmda* (118), *tub*2 (75), *rpb*2 (50), *act* (44), ITS (8), and LSU (5) (Figure 5). The low PIV values of ITS and LSU confirm the inefficiency of these genomic regions in species discrimination.

The dataset using *tef*1, cmda, and tub2 shows an increase in the "Concordance Factor" for branches that are well-supported (Figure 6).

4. DISCUSSION

Among the four species examined in our study, *S. siegesbeckiae* and *S. vernoniae* are being reported for the first time in Brazil. *S. crepidis* has previously been reported on *Crepis japonica* (Asteraceae) in Brazil, as well as other species within the *Crepis* genus (Vestergr, 1895; Radulescu et al., 1973; Adamska, 2001; Pereira et al., 2002). This study represents the first record of this fungus infecting *Rhododendron simsii*, a member of the Ericaceae family. This finding suggests that host specificity, a

historically important criterion for the delimitation of *Septoria* species (Verkley et al., 2013; Quaedvlieg et al., 2013), is not a reliable criterion for the accurate identification of species.

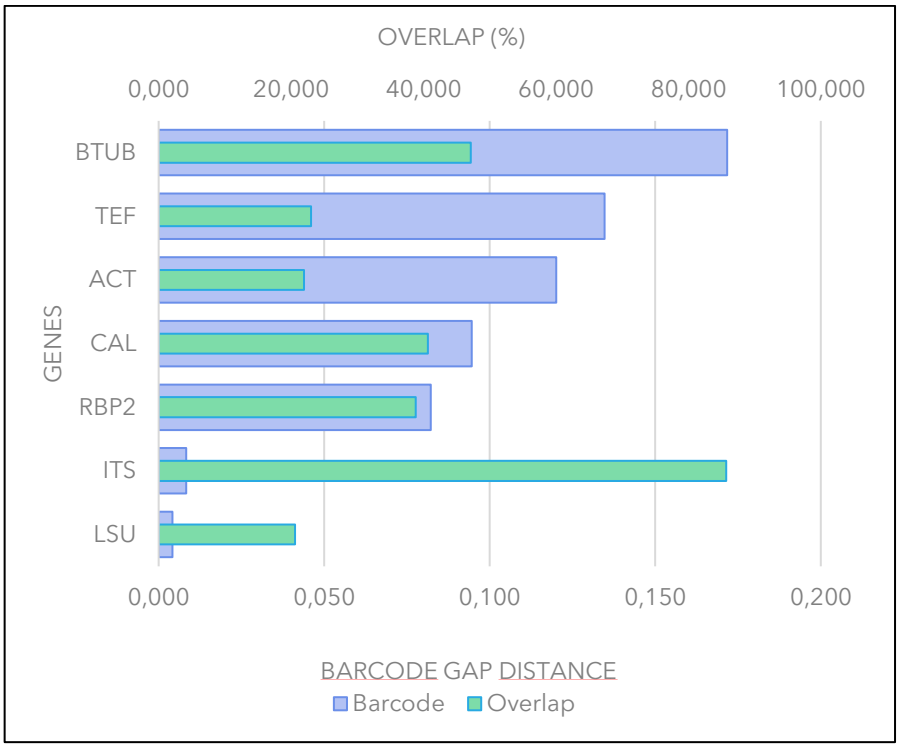

Figure 4. *Barcode gap* distance and *intra/inter-specific overlap distance* values for the most used genes for *Septoria* species delimitation. The blue bar indicates the barcode gap values and the green bars indicate the overlap value (%).

The species *S. siegesbeckiae* previously reported on *Sigesbeckia orientalis* (Asteraceae) (Siemaszko, 1923), was recorded on *Acanthospermum hispidum* (Asteraceae). However, *S. acanthospermi* was previously identified as the causal agent of Septoria leaf spot on *A. hispidum* (Sukapure & Thirumalachar, 1963). Morphological comparisons of *S. acanthospermi* and *S. siegesbeckiae* reveal notable similarities in conidium length (20—30 × 1—1.5 µm) and septation (Siemaszko, 1923; Sukapure & Thirumalachar, 1963). However, the molecular characterization of *S. acanthospermi* collected in India is essential to understand the diversity of *Septoria* species on *A. hispidum*.

Conyza canadensis (*Asteraceae*) has been reported as a host of *Septoria erigerontis* in Brazil (Esper et al., 2010; Duarte et al., 2016). The identification was based on host specificity and sequence comparison of the ITS region in the GenBank database. The isolates from *C. canadensis* formed a phylogenetically distinct clade of

the *S. erigerontis* and other known species (Figure 1). Therefore, a new species of *Septoria* will be proposed in accordance with the rules of the International Code of Nomenclature for Algae, Fungi, and Plants. Furthermore, this information confirms that different *Septoria* species can infect the same host.

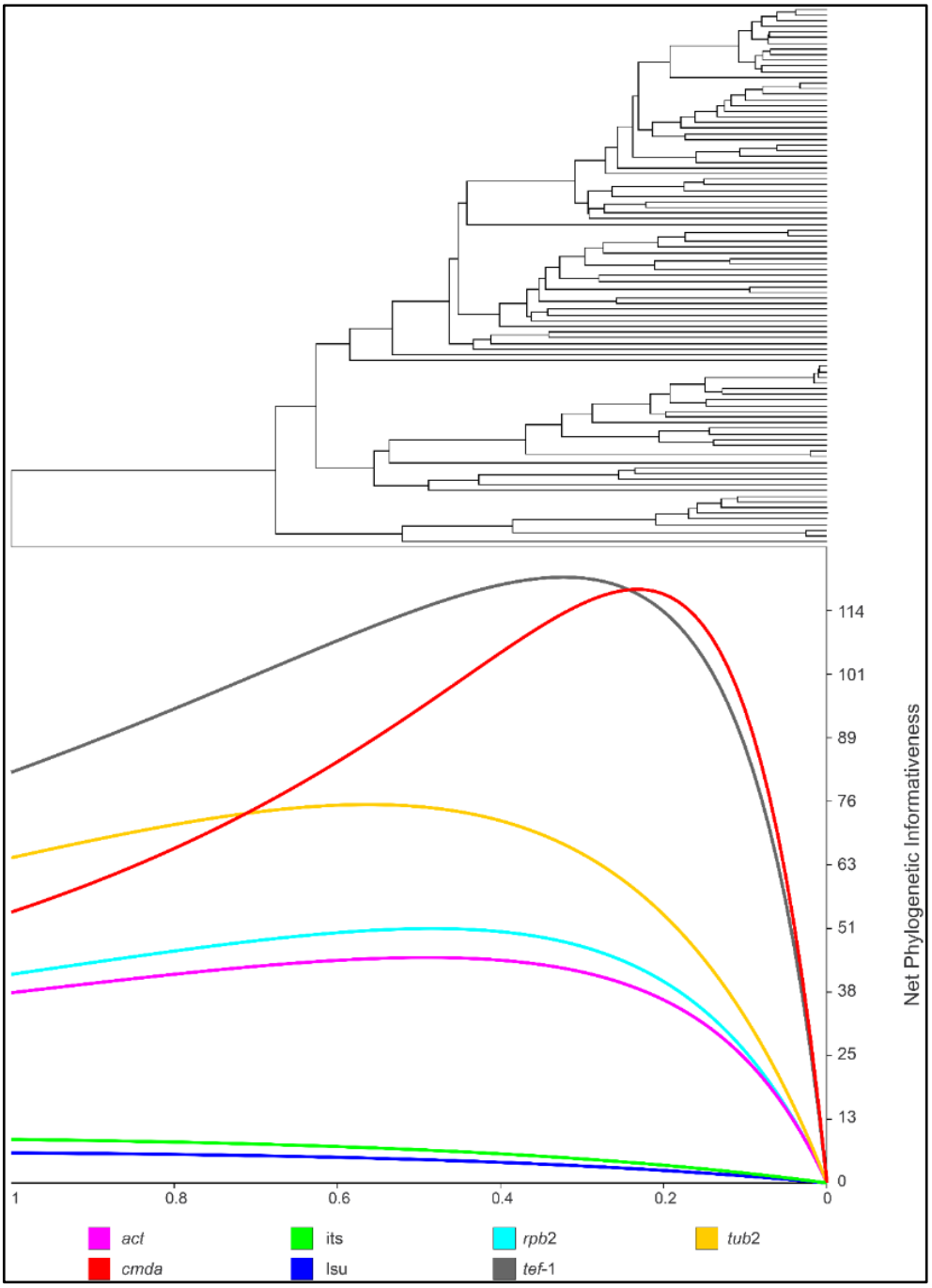

Figure 5. Ultrametric trees and net phylogenetic informativeness profiles of markers used for phylogenetic studies of *Septoria* species. Values on the X-axes correspond to the relative timescale (0—1) based on the root-to-tip distance. Values on the Y-axes represent net phylogenetic informativeness values in arbitrary units.

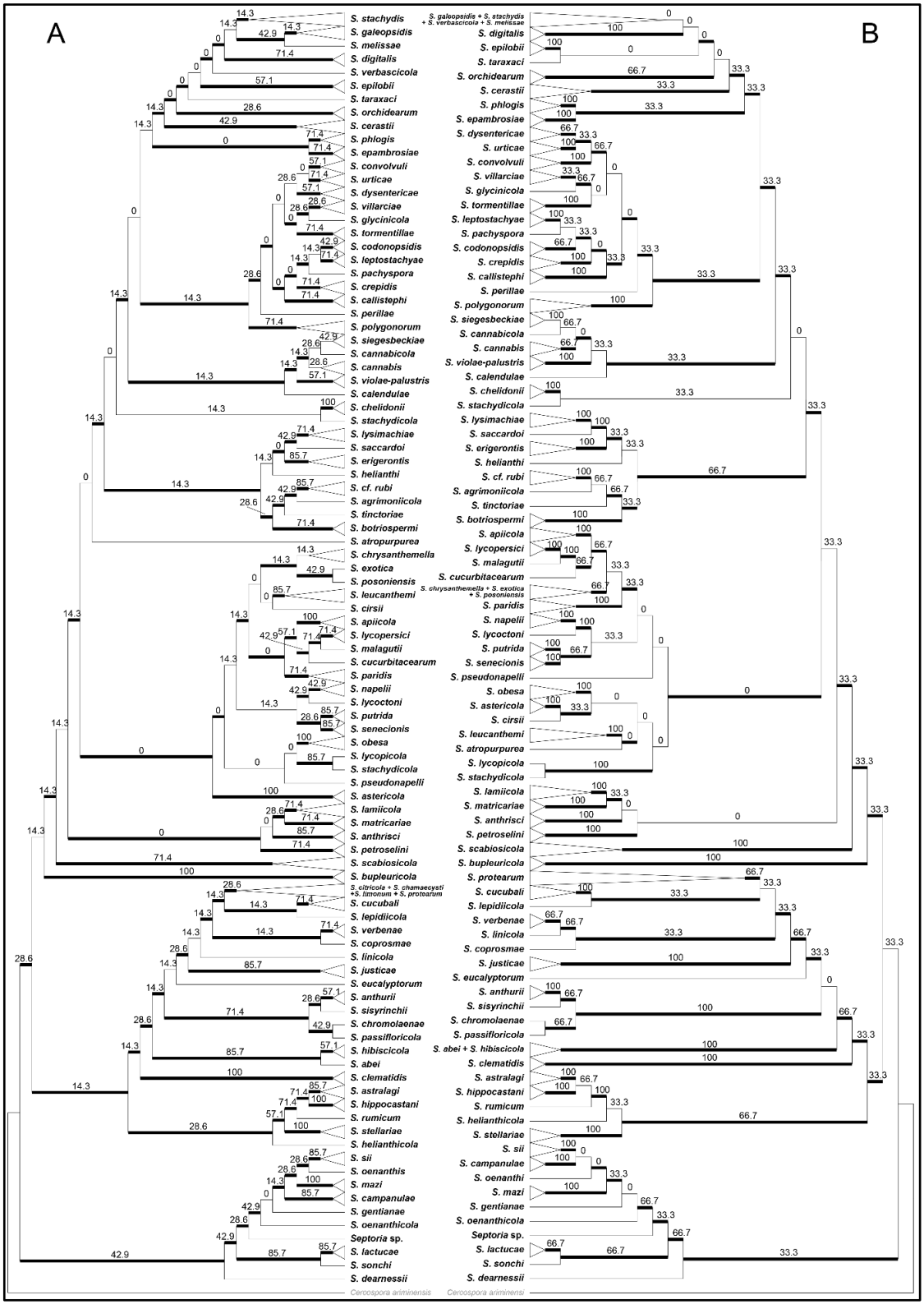

Figure 6. Maximum Likelihood (ML) trees of *Septoria* inferred from concatenated alignments. (A) All genes (*act*, *cmda*, ITS, LSU, *rpb*2, *tef*1 and *tub*2); (B) Three best genes (*cmda*, *tef*1 and *tub*2). Concordance factor (%) values are shown above the branches; each gene is represented by 14.3% in A and 33.3% in B. Branches well supported in the ML analyses (SH-arlt bootstrap support ≥ 80%) were thickened.

Septoria vernoniae was previously described on *Vernonia brasiliana* in Venezuela (Sydow, 1930), However, this species has not been molecularly characterized. Furthermore, none of the isolates obtained produced spores for morphological comparison. Therefore, once morphological comparisons can be made, a molecular description of *Septoria vernoniae* will be established. For this reason, the classification *S.* cf. *vernoniae* was assigned.

The discovery of *Septoria* on plants from different botanical families challenges traditional conceptions and highlights the importance of morphological, pathogenic, and molecular characterization for the accurate identification of species within this genus. A notable example is *S. protearum*, previously considered a complex of distinct species. However, analyses based on the sequencing of different genomic regions revealed that it is a single species with a broader host range (Verkley et al., 2013).

Furthermore, the delimitation of some species remains uncertain. Species delimitation should consider the patterns observed in the individual gene trees, not just the multilocus result. Moreover, many species have only a single molecularly characterized isolate, which complicates the differentiation between closely related species. This methodological limitation can lead to inaccurate identifications, highlighting the need for an integrated approach that considers multiple sources of evidence for a more reliable and accurate taxonomy.

We used multiple methods to evaluate the efficiency of genetic markers in identifying *Septoria* spp. These methods have its advantages and limitations, offering complementary insights into the ability of markers to distinguish species within the genus. To enhance the robustness of our analysis, we integrated these methods to identify the markers that best balance informativeness and phylogenetic resolution in *Septoria*.

Based on our results, the ITS and LSU regions exhibit a low capacity to delimit species within *Septoria,* which may weaken branch support in phylogenetic analyses (Verkley, 2013). When comparing the dataset consisting of the three best-ranked phylogenetic regions (*tef*1, *cmda* and *tub*2) with the complete set of genomic regions, we observe a general increase (with rare exceptions) in the "Concordance Factor" for well-supported branches (Figure 6). This increase can be attributed to the reduction of discordance caused by less informative genomic regions. These less informative regions tend to introduce uncertainties and ambiguities in phylogenetic analyses, while

the selection of more informative markers with greater resolving power results in more consistent trees with stronger phylogenetic support for the branches. The use of these three regions is sufficient for routine species identification, reducing economic and operational costs in species identification. However, for new species descriptions, all seven genomic regions may be necessary to ensure robust molecular data.

Despite using either seven genes or the three best-performing ones, some phylogenetic relationships remain unresolved, as shown by low internal branch support in both approaches. In these cases, it is essential to analyze the topology of the individual gene phylogenetic trees, as this approach provides more robust and reliable results for species identification by considering the variation and evolutionary relationships specific to each genomic region. Our study highlights how these factors, combined with locus selection and filtering, influence phylogenetic estimation in *Septoria*.

4. Conclusions

This study provides valuable insights into the taxonomy of *Septoria*, contributing to a more precise classification and a better understanding of the species diversity. Twenty-seven isolates obtained from hosts of two botanical families represented four species, including a new species. The host non-specificity confirms the inefficiency of this criterion for species delimitation. In addition, the phylogenetic informativeness analysis indicated that the *tef1*, *cmda* and *tub2* genomic regions are the most effective. Thus, sequencing these three regions is sufficient for routine identification. These results reinforce the importance of integrating both morphological and molecular approaches for a more accurate species identification.

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Suplementary Tables

Supplementary Table 1. Septoria isolates used in the study

CCUB	Species	Host	Family	City	State
3397	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Samambaia	Federal District
3398	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Tijucas	Santa Catarina
3403	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Brusque	Santa Catarina
3404	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Porto Belo	Santa Catarina
3413	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Porto Belo	Santa Catarina
3417	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Porto Belo	Santa Catarina
4546	*S.* cf *vernoniae*	*Vernonia polysphaera*	Asteraceae	Tijucas	Santa Catarina
3268	*S. crepidis*	*Crepis japonica*	Asteraceae	Undetermined	-
3269	*S. crepidis*	*Crepis japonica*	Asteraceae	Undetermined	-
3400	*S. crepidis*	*Crepis japonica*	Asteraceae	Tijucas	Santa Catarina
3402	*S. crepidis*	*Rhododendron simsii*	Ericaceae	Antonina	Paraná
3405	*S. crepidis*	*Crepis japonica*	Asteraceae	Antonina	Paraná
3410	*S. crepidis*	*Crepis japonica*	Asteraceae	Tijucas	Santa Catarina
3412	*S. crepidis*	*Crepis japonica*	Asteraceae	Registro	São Paulo
3416	*S. crepidis*	*Crepis japonica*	Asteraceae	Tijucas	Santa Catarina
3422	*S. crepidis*	*Crepis japonica*	Asteraceae	Registro	São Paulo
3565	*S. crepidis*	*Crepis Japonica*	Asteraceae	Vargem Bonita	Federal District
3566	*S. crepidis*	*Crepis Japonica*	Asteraceae	Vargem Bonita	Federal District
3567	*S. crepidis*	*Crepis Japonica*	Asteraceae	Taguatinga	Federal District
3570	*S. crepidis*	*Crepis Japonica*	Asteraceae	Gama	Federal District
4577	*S. crepidis*	*Crepis japonica*	Asteraceae	Tijucas	Santa Catarina
3265	*S. siegesbeckiae*	*Acanthospermum hispidum*	Asteraceae	Undetermined	-
3267	*S. siegesbeckiae*	*Acanthospermum hispidum*	Asteraceae	Carnaubal	Ceará
3270	*S. siegesbeckiae*	*Acanthospermum hispidum*	Asteraceae	Undetermined	-
4523	*S. siegesbeckiae*	*Acanthospermum hispidum*	Asteraceae	Camocim de São Felix	Pernambuco
4513	*Septoria* sp.nov.1	*Conyza canadensis*	Asteraceae	Sumidouro	Rio de Janeiro
4515	*Septoria* sp.nov.1	*Conyza canadensis*	Asteraceae	Sumidouro	Rio de Janeiro

Supplementary Table 2 Statistical models for Bayesian Inference and Maximum Likelihood analysis of multilocus phylogenetic trees based on seven genes (act, cal, ITS, LSU, rpb2, tef1, tub2).

Locus	ML model	BI model
act	*HKY+F+I+G4*	*HKY+I+G*
cmda	*TIM3+F+I+G4*	*GTR+I+G*
ITS	TIMe+I+R3	GTR+I+G
LSU	TIMe+I+R2	GTR+I+G
rpb2	*TPM3+I+G4*	*SYM+I+G*
tef1	TIM2+F+I+G4	GTR+I+G
tub2	TN+F+I+G4	GTR+I+G

Supplementary Table 3. Phylogenetic informativeness values (PIV) calculated using the PhyDesign.

Locus	PiMax	PIV
act	0,49	44
cmda	0,23	118
ITS	0,99	8
LSU	0,99	5
*rpb*2	0,48	50
*tef*1	0,32	120
*tub*2	0,56	75