Ramularia leaf spot: PCR-based methods reveal widespread distribution of *Ramulariopsis pseudogycines* and limited presence of *R. gossypii* in Brazil

Aline Suelen Silva
Universidade de Brasília
Marcelo Henrique Lisboa Renno
Universidade de Brasília
Ana Clara Ribeiro Quitania
Universidade de Brasília
Adalberto Correa Café-Filho
Universidade de Brasília
Robert Neil Gerard Miller
Universidade de Brasília
Alderi Emidio Araujo
Empresa Brasileira de Pesquisa Agropecuária
Danilo Batista Pinho (✉ danilopinho@unb.br)
Universidade de Brasília

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Abstract

Whilst Brazil is the fourth largest cotton producer globally, incidence of ramularia leaf spot (RLS) has decreased yield across all growing regions. In 2017-18 and 2018-19 growing seasons, ca. 300 fungal samples were collected from seven Brazilian states. Hyphal tip cultures were obtained for DNA extraction and amplification of partial sequences of the RNA polymerase II gene (*RPB2)*, 28S rRNA, the ribosomal DNA internal transcribed spacers (*ITS*), actin (*ACT)*, elongation factor (*EF1-α*) and histone H3 (*HIS3)* gene regions. Genetic diversity of the isolates was evaluated using fourteen molecular markers. Clade assignments based on the concatenated-sequence tree (*RPB2*, *LSU*, *EF1-α*, *ITS*, *ACT*, and *HIS3*) were identical to those in tree clades generated by *RPB2*-sequences, as well as in an *RPB2* haplotype network, in an ISSR $(TGTC)^4$ dendrogram, and based on morphological comparisons. In total, 252 out of 267 isolates were identified as *Ramulariopsis pseudoglycines*, indicating this species as the most widespread causal agent of cotton RLS in the Brazilian growing regions today. Validation of the ISSR $(TGTC)^4$ primer as a tool to study the diversity and distribution of *Ramulariopsis* species will make it possible to carry out extensive RLS sampling studies worldwide.

Introduction

Cotton (*Gossypium* spp.) is the world´s most cultivated fiber crop, mostly for the supply of raw materials for the textile industry, as well as for oil and protein extraction[1]. Since the 1990s, Brazil has ranked globally in fourth place in terms of cotton production, following a strong investment in the production technology and in the expansion of cultivated area, mainly in the Brazilian Cerrado[2,3,4,5] (a savannah-like region in the Brazilian Midwest).

Favorable environmental conditions during the growing season, combined with the cultivation of a limited number of cotton genotypes over very large areas, favors epidemics of ramularia leaf spot[4,6]. This disease was first reported in Paraguari, Paraguay in 1883[7], then soon after followed by a report from Alabama, USA in 1890[8]. Since then, the disease has been reported in more than 40 cotton producing countries[9,10]. Typically, ramularia leaf spot (RLS) is observed at the end of the cotton cycle and is therefore generally considered as a disease of secondary importance.

In Brazil, the disease was first reported by the Agricultural Inspection and Defense Service of São Paulo in 1919 and was considered of secondary importance until the 90's[9,11]. Currently, RLS is the major cotton disease in Brazil, with up to eight spray applications of fungicide required to reduce its negative effects on yield and cotton fiber quality[4,5,12].

Historically, *Ramularia areola* was the specific epithet used to designate the causal agent of RLS[13]. In 1961, this species was recombined to *Ramularia gossypii* (Speg.) Cif. and, due to the morphological similarities with the related genus *Ramulariopsis* (conidiophores severely branched at the base with terminal and lateral conidiogenic cells), the pathogen recombined again in 1993 to *Ramulariopsis*

gossypii (Speg.) Braun[14]. Later, a multigenic study including a representative number of isolates of *Ramularia* and allied genera revealed a new species, *Ramulariopsis pseudoglycines*, associated with RLS[15].

In Brazil, the etiology of the disease is so far inaccurate, given that most identifications are based on only few isolates or limited to the examination of morphological data[15,16,17]. Furthermore, it has been shown that a molecular perspective, associated with morphological data, is required to resolve plant pathogen species complexes, with this combined approach effective in revealing previously uncharacterized species affecting different crops[15,18,19]. For example, molecular characterization of isolates previously identified as *Ramularia eucalypti* based exclusively on morphological comparisons revealed a total of seven species associated with the eucalyptus leaf spot[20].

The diverse reactions of resistant and susceptible genotypes of cotton to different isolates of *Ramulariopsis* has been ascribed to the genetic variability of the pathogen[21]. Recently, an analysis of the genetic diversity of 16 isolates of *Ramulariopsis* revealed three genetic groups[22]. This information indicates a greater variability of the causal agent of the RLS than has so far been acknowledged. Nevertheless, there are still relatively few studies addressing the genetic diversity of *Ramulariopsis* associated to cotton in Brazil[4,21,22,23].

Accurate identification of the RLS pathogen in Brazil, as well as the relative abundancy of each causal agent, and respective geographic distribution, are particularly important for disease management recommendations, and are paramount for cotton breeding programs. The precise identification of the *Ramulariopsis* species in each growing area, combined with the phytosanitary measures, can minimize damage caused by RLS.

Here, *Ramulariopsis* isolates collected in the main cotton growing regions in Brazil were morphologically and molecularly characterized. In addition, a PCR-based method was developed to distinguish between isolates of *R. gossypii* and *R. pseudoglycines*, aiding breeders and plant pathologists in cotton disease resistance development and fungicide resistance avoidance.

Results

Sampling and Isolates

Symptomatic leaf samples were collected from 24 production fields representing seven Brazilian states (Fig. 1). Naturally-occurring symptoms included light green to yellow-green lesions delimited by the veinlets, giving them an angular or irregular shape, with white powdery sporulation on both sides of the leaves. Under favorable disease conditions, the lesions coalesced, become chlorotic and then necrotic, and often followed by severe defoliation (Fig. 2). Two hundred and sixty-seven *Ramulariopsis* isolates (Supplementary Table S1) were obtained in the Brazilian states of Bahia (n=44), Distrito Federal (n=32), Goiás (n=31), Maranhão (n=30), Mato Grosso (n=87), Mato Grosso do Sul (n=39) and Paraíba (n=4).

Phylogenetic analysis

RPB2 amplicons were obtained for 267 isolates, generating sequences of approximately 930 bp, which were deposited in GenBank under accession nos. MZ039858 to MZ040124. The *RPB2* matrix included 271 taxa (267 isolates from this study and 4 taxa from GenBank), composed of 847 sites (740 conserved) and 91 parsimony-informative characters. The BI tree was reconstructed using the GTR nucleotide substitution model. The *RPB2* tree (Fig. 3) showed that the *Ramulariopsis* isolates were grouped into two distinct clades (the nucleotide matrices and phylogenetic tree are available in TreeBASE; study number S28159). Clade II gathered most (94.4%) of the isolates from the states of Bahia (44), Distrito Federal (21), Goiás (31), Maranhão (30), Mato Grosso (87) and Mato Grosso do Sul (39). The remaining 15 isolates (5.6 %) were grouped in Clade I, with 11 isolates from the Distrito Federal and four from the state of Paraíba.

To correctly delimit the *Ramulariopsis* isolates at the species level, a multilocus approach was adopted using the *RPB2*, *LSU*, *EF1-α*, *ITS*, *ACT*, and *HIS3* sequences. A total of 21 taxa (Supplementary Table S2) were included in the BI and ML phylogenetic analyses. The *RPB2*, *LSU*, *EF1-α*, *ITS*, *ACT*, and *HIS3* individually aligned data sets were 942, 873, 1112, 182, 156, and 346 bp in length, respectively (single gene trees are available in TreeBASE; study number S28159). The concatenate alignment comprised 3611 characters, with 3316 and 281 conserved and variable sites, respectively. Also, 279 sites were determined as phylogenetically informative. The Bayesian phylogenetic tree was reconstructed considering the best nucleotide substitution model for each partition in the concatenate data, GTR (*RPB2*), HKY (*EF1-α, HIS3*, *ITS*, LSU) and K80 (*ACT*). The *Ramulariopsis* isolates reported here were grouped into two distinct phylogenetic clades (Fig. 4), corresponding to *R. gossypii* (clade I) and *R. pseudoglycines* (clade II).

Morphological characterization

The morphological characteristics of the isolates belonging to *R. gossypii* and *R. pseudoglycines* in the concatenated tree matched well with the description of each species (Table 2). The long conidiophores of *R. pseudoglycines* are readily distinguished from the short conidiophores of *R. gossypii* by visualization under stereomicroscope or light microscopy. These morphological differences were recorded by SEM and are illustrated here for the first time (Fig. 5).

Genetic characterization

A high interspecific polymorphism and low intraspecific polymorphism was observed among *Ramulariopsis* isolates for all 14 markers. The dendrograms based on the binary matrix produced from the band patterns generated with all markers separately were used to analyze the interspecific diversity of *Ramulariopsis* (data not shown). The ISSR $(TGTC)^4$ molecular marker was selected to estimate the interspecific diversity due to its´ simplicity for species discrimination. Only one amplicon of approximately 600 bp was observed for *R. pseudoglycines,* while two amplicons of approximately 1000 and 2000 bp were observed for *R. gossypii* (Fig. 6). To confirm the efficacy of this molecular marker in

distinguishing these two *Ramulariopsis* species from other contaminants or fungal pathogens, amplifications were also performed with *Fusarium* sp., *Colletotrichum* sp., *Talaromyces* sp. and *Baudoinia* sp., demonstrating the efficiency of the molecular marker to distinguish the two species of *Ramulariopsis*. The dendrogram of 267 isolates of *Ramulariopsis* generated by amplification of the $(TGTC)^4$ primer (Fig. 7) revealed two distinct clades corresponding to the clades previously observed in the phylogenetic analysis.

Intraspecific diversity

Analysis of intraspecific genetic variability revealed four distinct haplotypes among the 267 *Ramulariopsis* sequences (Supplementary table S1). The *RPB2* haplotype network (Fig. 8) revealed two distinct clades corresponding to the clades already observed in the phylogenetic tree and the dendrogram. The first clade is restricted only to *R. gossypii* isolates from the Distrito Federal and Paraíba. The second clade is represented by isolates of *R. pseudoglycines* from multiple geographic regions, including all sampled locations. Two out of three *RPB2* haplotypes were represented by only one isolate of *R. pseudoglycines.* All isolates of *R. gossypii* (n=15) grouped into a single haplotype (H3) while 250 out of the 252 isolates belong to the most frequent *R. pseudoglycines* haplotype (H1), indicating strong prevalence of clonal populations.

Discussion

Large-scale studies that investigate RLS etiology and the genetic variation of the causal agent are scarce in the literature. The center of origin of cotton has not been determined, but the main centers of diversity are distributed among Central America, Africa, Arabia and Australia[24]. In most of the countries within these regions, RLS is considered as a disease of secondary importance, in contrast to Brazil.

Global cotton yield has been affected by *R. gossypii* since 1883[7]. Historically, this species is most widespread and economically important in Brazil, although it has been mostly identified using only morphological data[4,15]. Although conidiophore length is useful for separating *Ramulariopsis* species, taxonomic expertise is required. Interestingly, several isolates previously putatively identified as *R. gossypii* were molecularly identified as *R. gossypii* and *R. pseudoglycines*[15]. Comparison of *ITS* sequences of the *Ramulariopsis* isolates deposited in the GenBank (the nucleotide matrices and phylogenetic tree are available in TreeBASE; study number S28159) revealed that *R. gossypii* and *R. pseudoglycines* were described in both previous studies[17,25], but the prevalence of each species was not determined, due to the limited number of isolates.

Here, the molecular identification of the *Ramulariopsis* isolates causing RLS on cotton using a polyphasic approach confirmed the existence of *R. gossypii* and *R. pseudoglycines* in Brazil. In total, 252 out of 267 isolates were identified as *R. pseudoglycines*, indicating this species as the most widespread causal agent of cotton RLS in the Brazilian growing producing regions today. Additionally, isolates of *R. gossypii* were restricted to small and isolated farms located in the Distrito Federal and the state of Paraíba, while

isolates of *R. pseudoglycines* were obtained from all sampled locations, and all extensive farms in the Brazilian Cerrado.

The clade assignments based on the concatenated-sequence tree (*RPB2*, *LSU*, *EF1-α*, *ITS*, *ACT*, and *HIS3*) were identical to those generated by *RPB2*-sequences trees, the *RPB2* haplotype network, the ISSR $(TGTC)^4$ dendrogram, and the morphological comparisons. The most widely employed genomic regions for *Ramulariopsis* DNA-based identifications have been based upon the *ITS* sequences, given both their high copy number and easy amplification, and the availability of universal primers. However, for various fungi, the *RPB2* molecular marker has been proposed in place of the *ITS* sequences, due to the lack of resolution in the latter and the potential presence of non-homologous *ITS* copies in individual fungal genomes[26].

On a molecular level, *RPB2* sequences are recommended for accurate molecular identification of *Ramulariopsis* isolates, given its universal application, speed, and the presumption that this molecular marker safely approximates taxonomic expertise. However, this technique is laborious, expensive, and requires time and knowledge of phylogenetic analysis for identifying the species[27]. The desire for rapid, automated approaches, such as that obtained here using the ISSR $(TGTC)^4$ primer, indicates that the *RPB2* region can potentially be used for easy and inexpensive diagnosis and detection assays.

This study showed that the ISSR $(TGTC)^4$ primer can be used for accurate molecular identification of *Ramulariopsis* isolates, facilitating large scale surveys of the distribution of species and monitoring of epidemics. Prior to this study, the diversity among isolates of *Ramulariopsis* was verified through ERIC- and REP-PCR profiles for Brazilian isolates[22] or RAPD profiles for Indian isolates[28], although in both those studies, only a few isolates were analyzed and accurate identification of the species was not determined.

Considering the wide distribution of haplotype H1 of *R. pseudoglycines*, there is evidence for a predominant clonal lineage occuring in Brazil, indicating the existence of a highly efficient mechanism of dispersion over long distances. Although RLS caused by *R. gossypii* has been recognized for a long time, *R. pseudoglycines* seems to be firmly prevalent amongst the cotton-producing regions today. When comparing the morphology of *Ramulariopsis* specimens from earlier studies[4,16], the morphological characteristics matched well with the description of *R. gossypii*. We believe that with the cultivation of few cotton genotypes in extensive areas in the Brazilian Cerrado, the population of *R. pseudoglycines* has also increased, becoming one of the most important pathogens negatively impacting cotton production in Brazil.

This is the first large-scale study that investigated the diversity of *Ramulariopsis* isolates associated to cotton. Validation of the ISSR $(TGTC)^4$ primer as a tool to study the diversity and distribution of *Ramulariopsis* species will make it possible to carry out extensive RLS sampling studies worldwide. Finally, the correct identification of the RLS causal agent and its´ geographical distribution is essential for

predicting resistance breakdown, guiding pesticide regimes and the development of disease resistant genotypes.

Methods

Sampling and isolation

Cotton leaves showing typical symptoms of RLS were collected in the 2017-18 and 2018-19 growing seasons from 24 commercial fields in the Brazilian states of Bahia, Distrito Federal, Goiás, Maranhão, Mato Grosso, Mato Grosso do Sul and Paraíba (Fig. 1).

Fungal isolation into pure culture was carried out by the direct method[29] in Petri dishes containing water-agar (WA) medium (20 g/L of agar). After 14 days of growth in WA, pure cultures were established by transferring a fragment of a hyphal tip to a new Petri dish containing malt extract (ME) medium (20 g/L malt extract and 20 g/L agar).

Isolates (Supplementary Table S1) were deposited in the Coleção de Culturas da Universidade de Brasília (CCUB; Brasília, Brazil) and stored at 18 ± 1 ° C in sterile water[30], 10% (v / v) sterile glycerol, and half potato-dextrose-agar (500 mL/L potato broth, 20 g/L agar and 20 g/L dextrose) slopes covered with sterile mineral oil.

DNA extraction

Four mycelial discs (5 mm in diameter) were removed from the margin of 20-day old pure cultures on ME and transferred to 250 mL conical flasks containing 50 mL of potato dextrose broth with addition of streptomycin (500 mL/L potato broth, 20 g/L dextrose, 100 µg/mL streptomycin) and incubated at 25 ° C, with a 12 h photoperiod.

After seven days growth, the developed mycelium was recovered on filter paper and transferred to 1.5 mL microtubes containing 30 µL of Tris-EDTA (TE) buffer, four metal beads (2.8 mm), and 600 mL of Nuclei Lysis Solution (Promega®). Total DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega®) according to the manufacturer's instructions. Total DNA preparations were analyzed via 1% agarose gel electrophoresis, stained with GelRed (Biotium R), and visualized under UV light. The DNA samples were stored at −20◦C.

Amplification and sequencing

Partial sequences of the second largest RNA polymerase II subunit (*RPB2*) were amplified using the specific PCR primers shown in Table 1, with this genomic region employed as the primary barcode for identification of *Ramulariopsis* species, given the high PCR success rate and easy alignment of the nucleotide sequences. To assign definite species demarcations for the *Ramulariopsis* isolates, partial nucleotide sequences of five nuclear genes, namely: 28S rRNA (*LSU*), the internal transcribed spacers of the ribosomal DNA (*ITS*), actin (*ACT*), elongation factor (*EF1-α*), and histone H3 (*HIS3*) were obtained

from representative isolates of different clades and locations preliminarily identified based on *RPB2* sequence data (Fig. 3). The primers employed are listed in Table 1, with respective annealing and extension parameters. The PCR mixtures consisted of 6.25 µL of MyTaq PCR Master Mix (2 ×), 0.3 µL of each primer (Table 1), 1 µL of genomic DNA (25 ng / µL) and 4.65 µL of ultrapure water. The cycling conditions were: Initial denaturation at 95 ºC for 1.5 min, followed at 35 cycles at 95 ºC for 20 s; annealing and extension according to Table 1 and a final extension at 72 ° C for 5 min. The PCR products were purified and bidirectionally Sanger-sequenced.

Phylogenetic analyses

To determine to which *Ramulariopsis* species each isolate shared the highest nucleotide identity, the partial nucleotide sequences and the BLASTn algorithm were used to search the NCBI-GenBank nonredundant nucleotide database. A Bayesian phylogenetic tree was initially reconstructed using the *RPB2* sequences from the 267 isolates characterized here, and four representative isolates of *Ramulariopsis*. The ex-epitype CBS 141099 of *R. gossypii* was used as an outgroup. Also, phylogenetic trees were individually inferred from each genomic region analyzed here. Multiple sequence alignments were obtained with MAFFT v7[31]. Finally, Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic trees were reconstructed using the concatenate data (RPB2, LSU, EF1-α, ITS, ACT, and HIS3). For BI, the best nucleotide substitution models were determined, for each partition, with MrModeltest. The CIPRES web portal[32] was used to run MrBayes v3.2.1[33]. The Markov Chain Monte Carlo (MCMC) analysis was run with a total of 10 million generations, sampling every 1,000 generations. The convergence of the log likelihoods was confirmed using TRACER v1.7.1[34] . The first 25% of the sampled trees were discarded as burn-in, with the posterior probability (PP) values calculated with the remaining trees. The ML tree was reconstructed using RAxML v.8[35], accessed through the CIPRES web portal[32], assuming a general time reversible (GTR) nucleotide substitution model with a gamma (G) rate of heterogeneity, and 1,000 bootstrap replicates. The phylogenetic trees were visualized and edited in FigTree v1.4[36] and Inkscape.

Light microscopy and SEM morphological characterization

For morphological characterization, specimens were initially observed with a Leica (Leica Biosystems, Nussloch GmbH, Nussloch, Germany) 205C stereomicroscope. The microscopical characteristics were analyzed by mounting asexual structures in clear lactoglycerol, and 50 measurements for each morphological parameter were carried out at a magnification of × 1,000 using a Leica DM2500 light microscope equipped with a Leica DFC 490 digital camera, coupled to a computer containing the Leica Qwin-Plus software. The morphological characteristics of the isolates were compared with the description of *R. gossypii* and *R. pseudoglycines*[14,15].

For examination on a scanning electron microscope (JOEL JSM-700 1F model), fragments of symptomatic dry leaves were fixed in 10 mm diameter copper stubs with double-sided carbon tape and coated with 25 mA gold, 1.10-2 mbar, for 2.5 minutes.

Genetic characterization

Seventeen isolates of *R. gossypii* (n=3) and *R. pseudoglycines* (n=14) were subjected to characterization with different molecular markers (CIIRAP1-4, CIIRAP2-4, REP, ERIC, BOX, M13, N21, CAG5, GA8, GACAC3, TGTC4, GATA4, GTG5 and GACA4) which are typically highly polymorphic and useful in analysis of genetic variability of fungi[22,37,38].

The PCR amplifications were performed in a final volume of 12.5 µL: 6.25 µL of MyTaq PCR Master Mix (2 ×), 2.5 µL of primer, 1 µL of genomic DNA (25 ng / µL) and 2.75 µL of ultrapure water. Different volumes of primer were used for REP and ERIC (0.5 µl), and BOX (1 µl) molecular markers, with a final reaction volume again adjusted to 12.5 µL. The PCR conditions for each molecular marker are shown in references listed in Table 1. Each amplification was repeated at least twice in separated assays.

The amplified products were evaluated as presence (1) or absence (0) of bands and recorded in a binary matrix. This matrix was added to the PAST3 software[39], where the Jaccard similarity index was calculated for each combination of two samples. From the similarity index, dendrograms were constructed according to the method of unweighted pair group method with arithmetic mean (UPGMA).

Intraspecific diversity

To characterize intraspecific genetic diversity of *R. pseudoglycines* and *R. gossypii*, an analysis of haplotypes was performed using the RPB2 sequences of the 267 isolates. Haplotype identification was performed using the program DnaSP ver. 5.10.1[40]. A haplotype network to visualize the relationships among haplotypes representing seven Brazilian states was reconstructed using NETWORK 4.5.0.2 (Fluxus Technology Ltd.), with gaps and missing data excluded[41].

Declarations

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by A.S.S., M.H.L.R., A.C.R.Q. and D.B.P. Conceptualization and review and editing were performed by A.C.C-F, A.E.A. and R.N. G.M. The first draft of the manuscript was written by A.S.S. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated in this study can be found in Genbank: MZ039858-MZ040124, and xxxxxxxx-xxxxxxxx; Treebase: S28159. The results obtained in this study are included in the contents of this report.

Author information

Affiliations

Graduate Programme in Plant Pathology, Universidade de Brasília, Brasília, DF, 70910-900, Brazil

Danilo Batista Pinho (orcid: 0000-0003-2624-302X), Aline Suelen da Silva (orcid: 0000-0002-0550-5847), Marcelo Henrique Lisboa Rennó (orcid: 0000-0001-5195-9930), Ana Clara Ribeiro Quitania (orcid: 0000-0002-8166-7127), Adalberto Corrêa Café-Filho (orcid: 0000-0002-5204-2961), Robert Neil Gerard Miller (orcid: 0000-0002-5798-4552).

Embrapa Algodão, Campina Grande, PB, Brazil

Alderi Emídio de Araújo (orcid: 0000-0003-0257-1539)

Ethics declarations

Conflicts of interest

The authors declare no conflict of interest.

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Tables

Table 1. Primers selected for phylogenetic analysis and inter and intraspecific diversity analysis of *Ramulariopsis* isolates.

Locus [1, 2]	Primer Name	Sequence 5'→ 3'	Orientation	References	(Anelling/ Extension)
Elongation factor 1-α (EF1α) [1]	EF1F	TGC GGT GGT ATC GAC AAG CGT	Forward	Jacobs *et al.*[42]	54°-45''/72°-45''
	EF2R	AGC ATG TTG TCG CCG TTG AAG	Reverse	Jacobs *et al.*[42]	54°-45''/72°-45''
Histone H3 (HIS3) [1]	CYLH3F	AGG TCC ACT GGT GGC AAG	Forward	Crous *et al.*[18]	60°-45''/72°-30''
	CYLH3R	AGC TGG ATG TCC TTG GAC TG	Reverse	Crous *et al.*[18]	60°-45''/72°-30''
28S nrRNA (LSU) e Internal transcribed spacer (ITS) [1]	V9G	TTA CGT CCC TGC CCT TTG TA	Forward	De Hoog & Ende[43]	53°-45''/72°-45''
	LR5	ATC CTG AGG GAA ACT TC	Reverse	Vilgalys & Hester[44]	53°-45''/72°-45''
Actin (ACT) [1]	ACT-512F	ATG TGC AAG GCC GGT TTC GC	Forward	Carbone & Kohn[45]	58°-25''/72°-25''
	ACT-783R	TAC GAG TCC TTC TGG CCC AT	Reverse	Carbone & Kohn[45]	58°-25''/72°-25''
RNA polymerase II second largest subunit (RPB2) [1]	RPB2-5f	GAY GAY MGW GAT CAY TTY GG	Forward	Liu *et al.*[46]	54°-45''/72°-45''
	7cR	CCC ATR GCT TGY TTR CCC AT	Reverse	Liu *et al.*[46]	54°-45''/72°-45''
REP [2]	REP1R-1	III ICG ICG ICA TCI GGC	Forward	Stern *et al.*[47]	44°-60"/72°-8'
	REP2-1	ICG ICT TAT CIG GCC TAC	Reverse	Stern *et al.*[47]	44°-60"/72°-8'
ERIC [2]	ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	Forward	Hulton *et al.*[48]	52°-60"/72°-8'
	ERIC2	AAG TAA GTG ACT GGG GTG AGC G	Reverse	Hulton *et al.*[48]	52°-60"/72°-8'
BOX[2]	BOXA1R	CTA CGG CAA GGC GAC GCT GAC G	-	Martin *et al.*[49]	53°-60"/72°-8'
Universal primer N21 [2]	N21	GGA TCC GAG GGT GGC GGT TCT	-	Bulat *et al.*[50]	55°-45"/72°-90"
Universal primer M13 [2]	M13	GAG GGT GGC GGT TCT	-	Vassart *et al.*[51]	50°-45"/72°-90"
IRAP [2]	CIIRAP1	CGT ACG GAA CAC GCT ACA GA	-	Santos *et al.*[52]	57,5°-30"/72°-120"
	CIIRAP2	AAT AAC GTC TCG GCC TTC AG	-	Santana *et al.*[38]	55,4°-30"/ 72°-120"
	CIIRAP4	CTT TTG ACG AGG CCA TGC	-	Santos *et al.*[52]	54,9°-30"/ 72°-120"
ISSR [2]	$(CAG)^5$	CAG CAG CAG CAG CAG	-	Rodrigues et al.[53]	60°-45"/72°-90"
	$(GA)^8$	GAG AGA GAG AGA GAG A	-	Andrea & Xitlali[54]	44°-45"/72°-90"
	$(GACAC)^3$	GAC ACG ACA CGA CAC	-	Weising *et al.*[55]	48°-45"/72°-90"
	$(TGTC)^4$	TGT CTG TCT GTC TGT C	-	Rodrigues *et al.*[53]	48°-45"/72°-90"
	$(GTG)^5$	GTG GTG GTG GTG GTG	-	Gente *et al.*[37]	58°-45"/72°-90"

Locus [1, 2]	Primer Name	Sequence 5'→ 3'	Orientation	References	(Anelling/ Extension)
	$(GACA)^4$	GAC AGA CAG ACA GAC A	-	Gente *et al.*[37]	50°-45"/72°-90"
	$(GATA)^4$	GAT AGA TAG ATA GAT A	-	Gente *et al.*[37]	35°-45"/72°-90"

[1] Molecular marker used for multigenic analysis.

[2] Molecular marker used to analyze inter and intraspecific diversity.

Table 2. Morphometric characteristics of *Ramulariopsis gossypii* and *R. pseudoglycines* on *Gossypium hirsutum* (Malvaceae).

SPECIES	Conidiophore µm	Conidia µm	Host
R. gossypii[1]	35−40 × 3	18−25 × 3−4	*G. hirsutum*
R. pseudoglycines[2]	121–175 × 2	6.5–8 × 2.5–3	*G. hirsutum*
R. gossypii CCUB 2939, CCUB 2883	25−44 × 2.5−3.5	13−19 × 3.5−4.5	*G. hirsutum*
R. pseudoglycines CCUB 2269, CCUB 3304	74,5–138.5 × 2.5–3	11–21.5 × 3–3.5	*G. hirsutum*

[1](Spegazzini, 1886); [2](Videira et al., 2016).

Figures

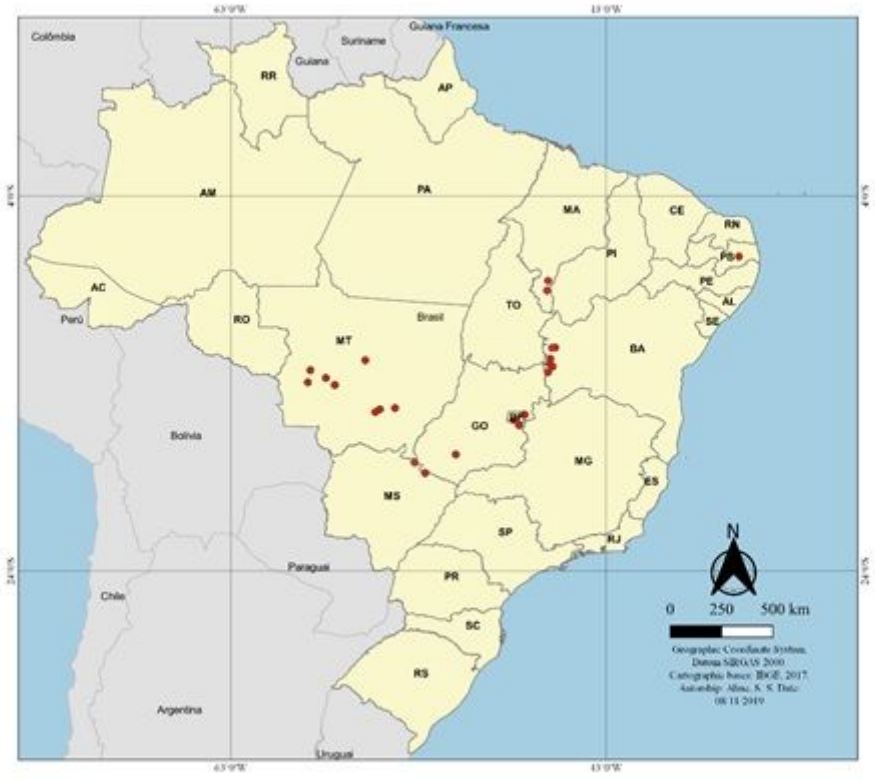

Figure 1

Map of Brazil showing the distribution of states and different collection points of Ramulariopsis isolates.

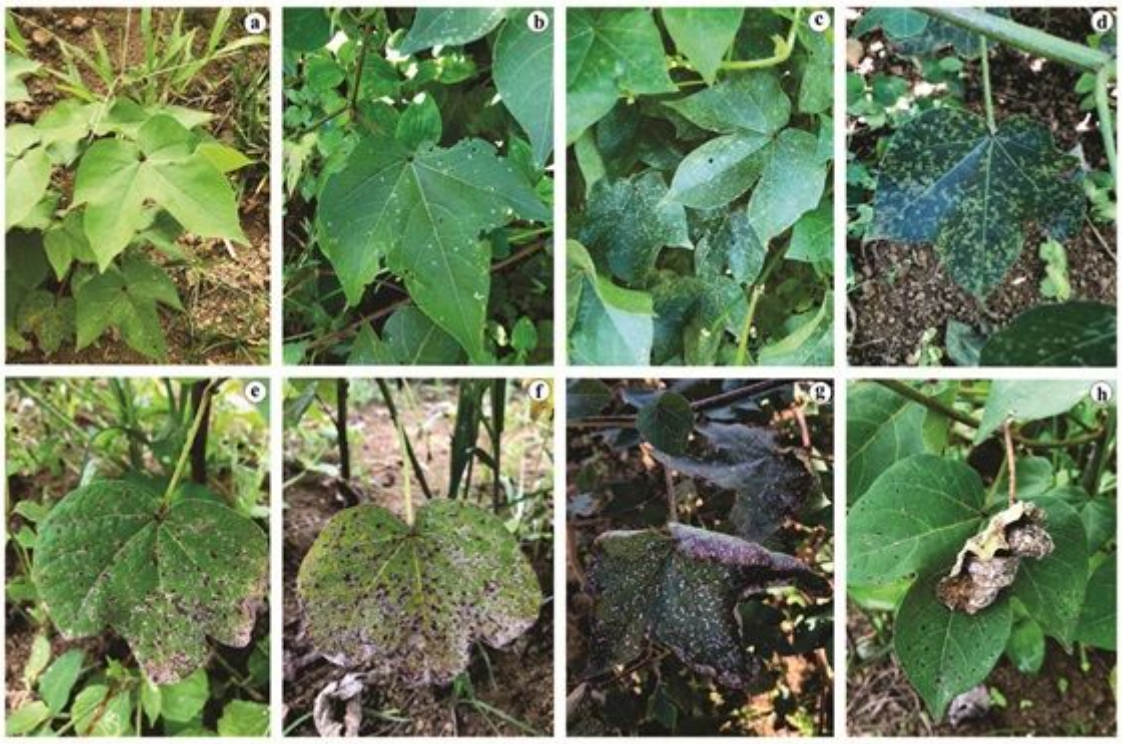

Figure 2

(A-H) Ramularia leaf spot symptoms in cotton. A. Healthy leaves observed in the field. B, C, D. Initial symptoms; Early sporulation of Ramulariopsis on adaxial sides of cotton leaf; E, F. Late sporulation on necrotic lesions on the upper surface of cotton leaf; G. Necrotic lesions covering the leaf. H. Fallen leaf with advanced necrotic lesions.

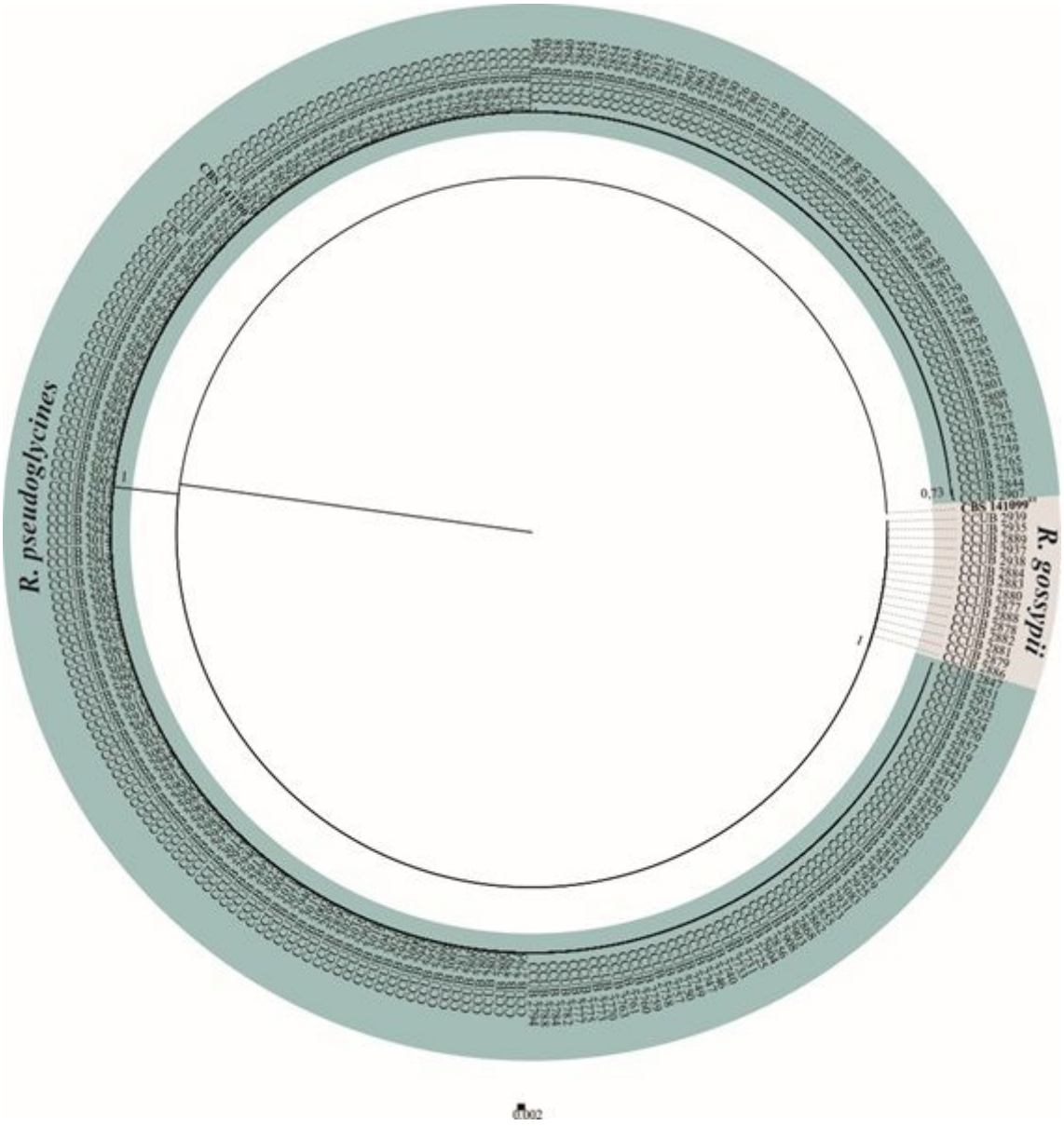

Figure 3

Bayesian phylogenetic tree based on RPB2 sequences of Ramulariopsis species. Bayesian posterior probabilities and Maximum Likelihood bootstrap support values are indicated at the nodes, and the scale bar represents the number of expected changes per site. Ex-type isolates are highlighted in bold. The ex-type CBS 141099 of Ramulariopsis gossypii was used as outgroup.

Figure 4

Bayesian phylogenetic tree based on concatenate sequences (RPB2, LSU, EF1-α, ITS, ACT, and HIS3) of Ramulariopsis species. Bayesian posterior probabilities and Maximum Likelihood bootstrap support values are indicated at the nodes, and the scale bar represents the number of expected changes per site. Ex-type isolates are highlighted in bold. The type CBS 141100 of Ramulariopsis pseudoglycines was used as outgroup.

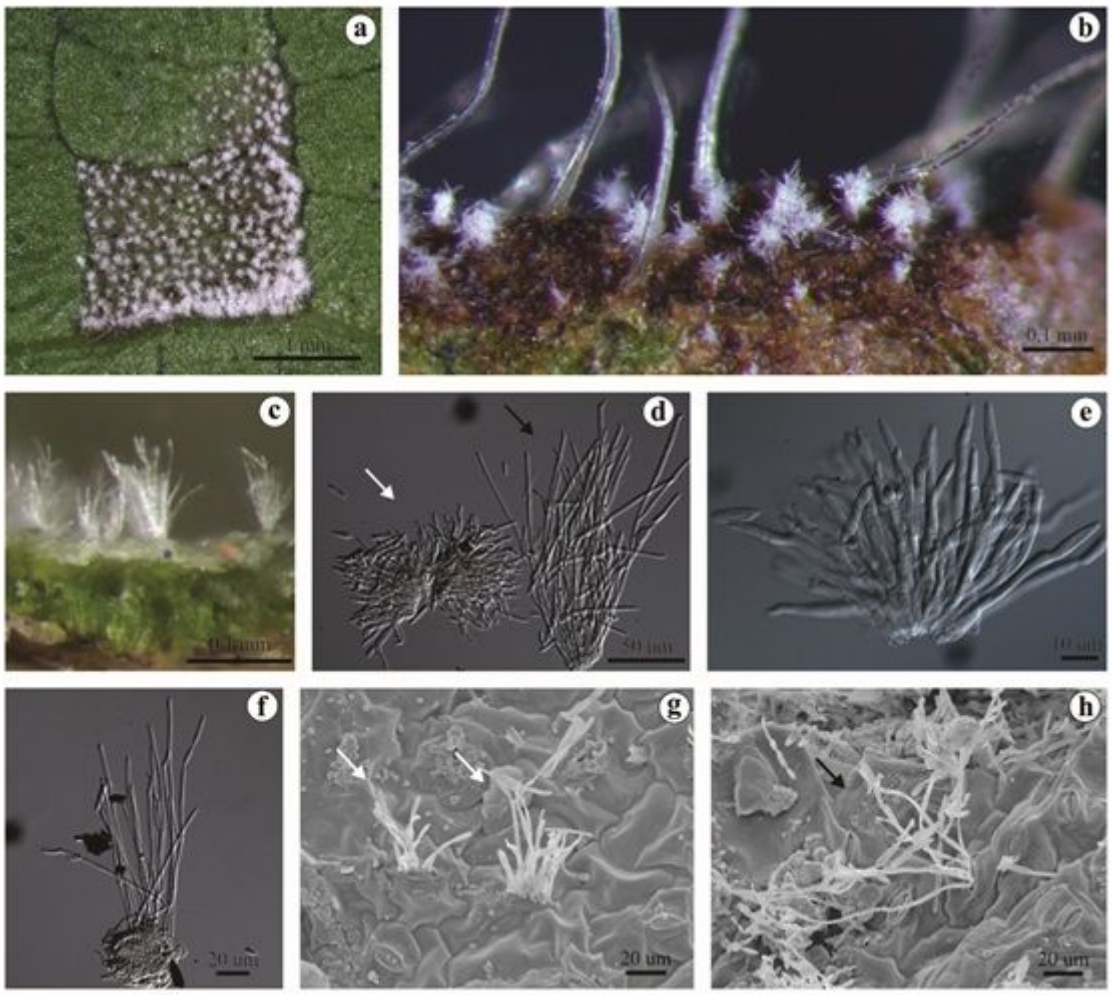

Figure 5

(A-H). Ramulariopsis spp. on leaves of Gossypium hirsutum. A. Lesion with signs of the fungus on the leaf abaxial face. B. Hyaline conidiophores of R. gossypii on the abaxial side of the leaves. C. Hyaline conidiophores of R. pseudoglycines on the abaxial side of the leaves. D. Hyaline conidiophores of R. gossypii (left) and R. pseudoglycines (right) viewed under light microscopy. E. Fascicle of R. gossypii formed by conidiophores with presence of hyaline conidia. F. Conidiophores of R. pseudoglycines. G. Conidiophores of R. gossypii visualized in SEM. H. Conidiophores of R. pseudoglycines visualized in SEM.

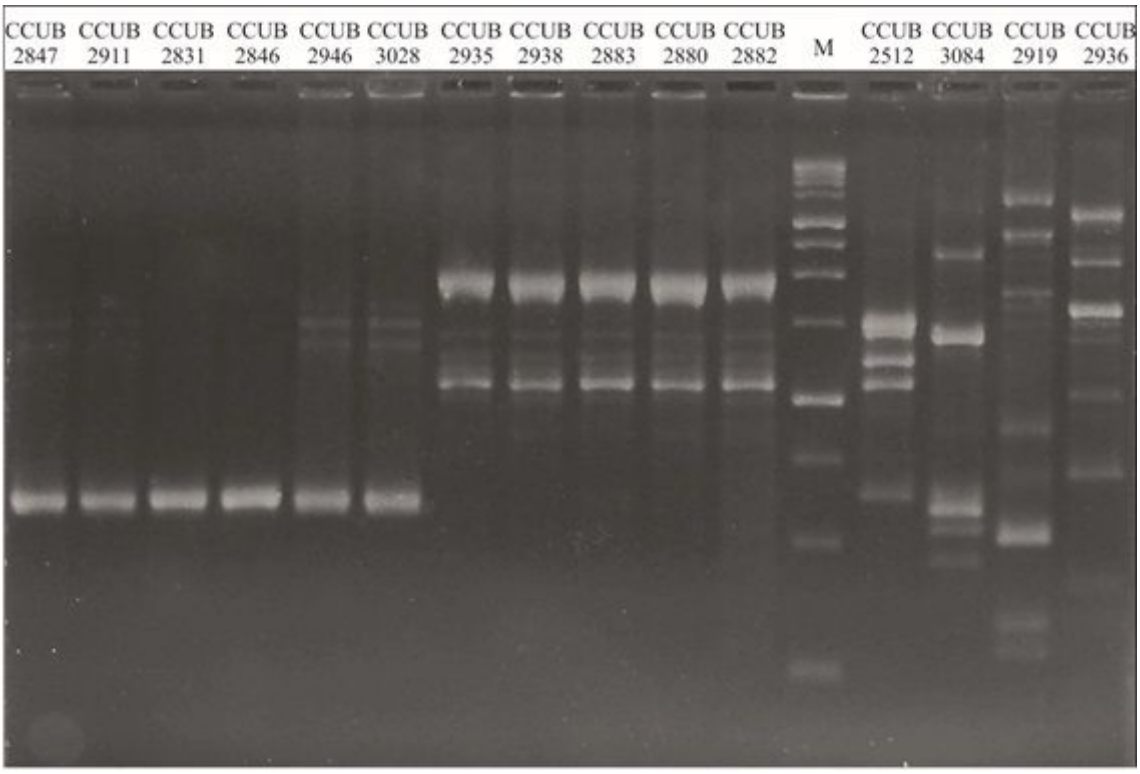

Figure 6

Amplicons visualized on 1.5% agarose gel from the amplification of isolates of R. pseudoglycines (CCUB 2847, CCUB 2911, CCUB 2831, CCUB 2846, CCUB 2946, CCUB 3028), R. gossypii (CCUB 2935, CCUB 2938, CCUB 2883, CCUB 2880 and CCUB 2882), Fusarium sp. (CCUB 2512), Colletotrichum sp. (CCUB 3084), Talaromyces sp. (CCUB 2919) and Baudoinia sp. (CCUB 2936), using the ISSR (TGTC) 4 primer. M = molecular marker 1Kb DNA Ladder Thermo Scientific.

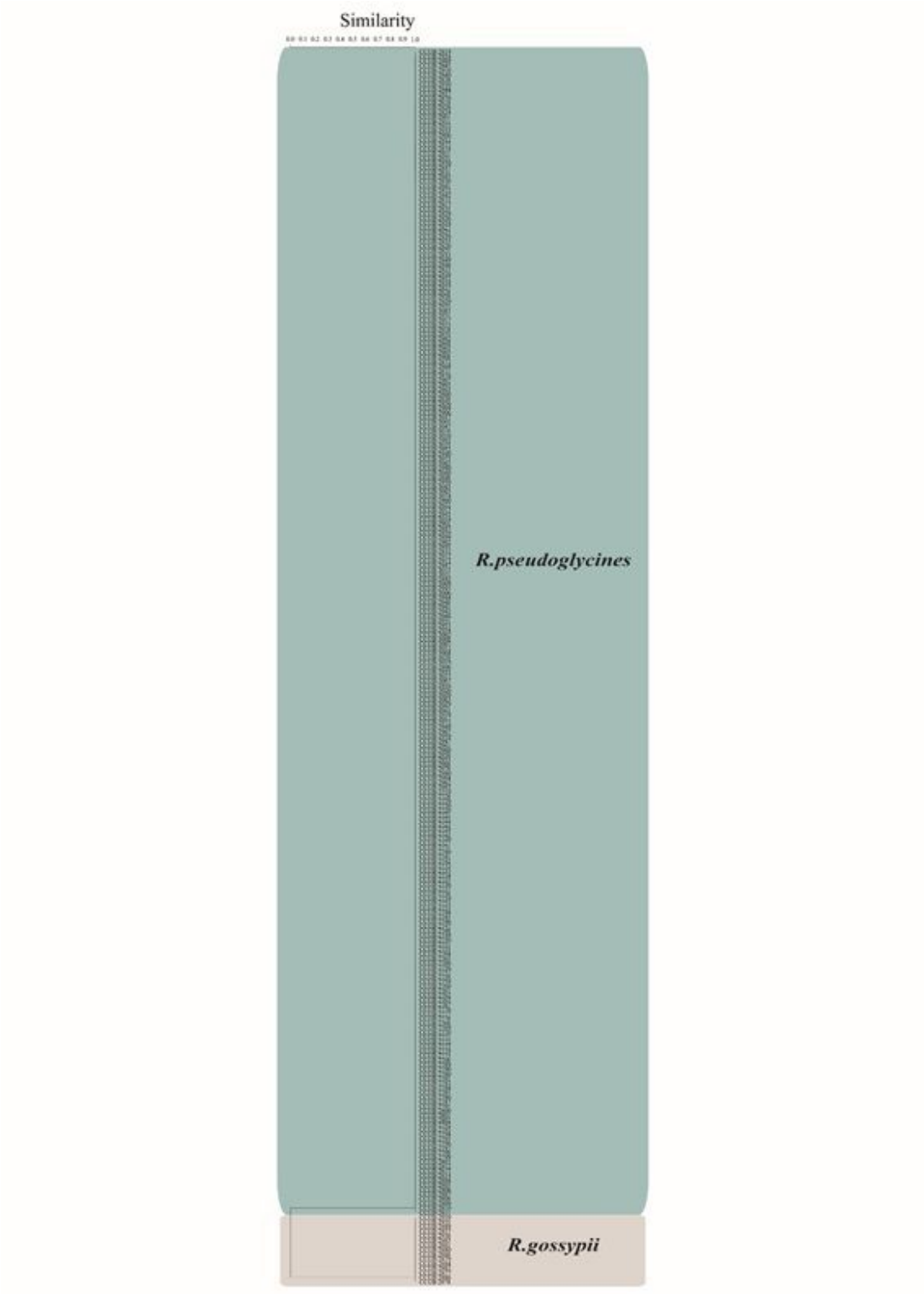

Figure 7

Dendrogram of 267 isolates of Ramulariopsis, generated by amplification with the ISSR (TGTC)4 primer, with the cutoff close to 70% of similarity. Genetic similarity pattern generated by the UPGMA method, based on Jaccard's coefficient.

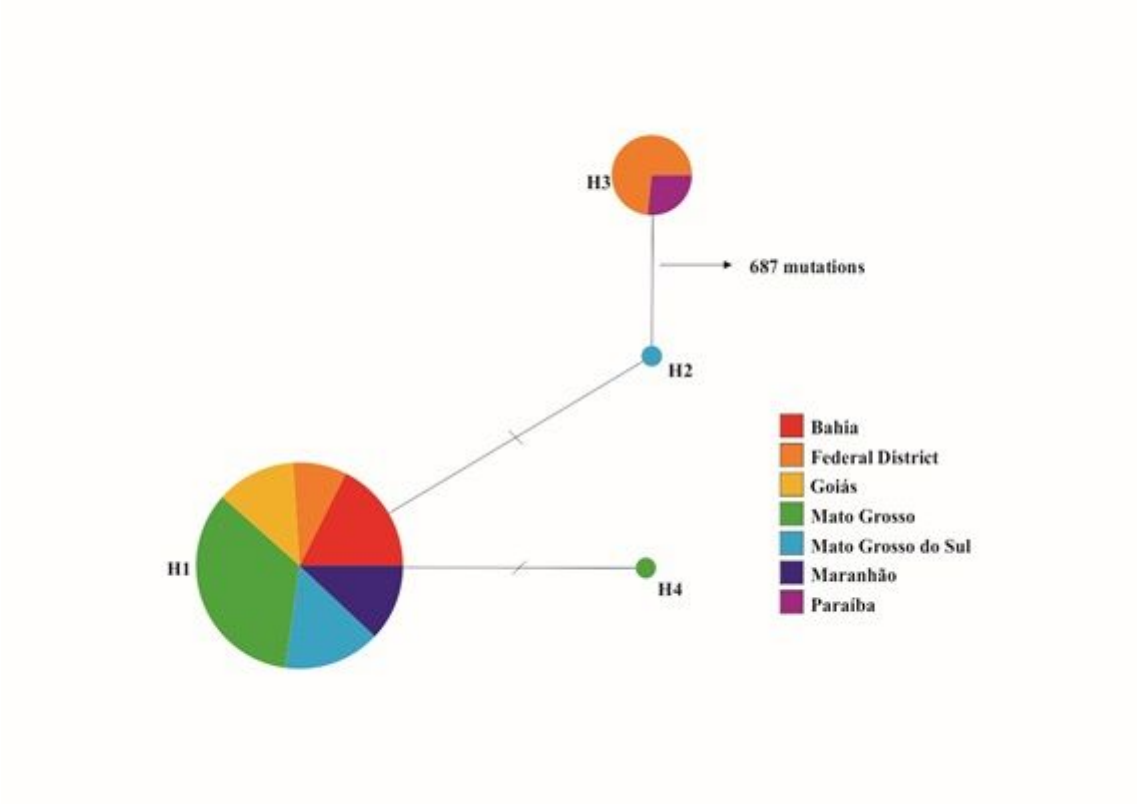

Figure 8

Haplotype network generated for RPB2 sequences representing seven Brazilian states using Network. Each circle represents a distinct haplotype, proportional in size to its´ frequency in the sample. Hatch marks along the network branches indicate hypothetical mutational steps not detected in the dataset. Geographic origin of isolates from each haplotype is proportionally represented in pie charts by different colours.

Supplementary Files

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