ARTIGOS

Phakopsora pachyrhizi is the major pathogen associated with soybean rust in Colombia

Anibal Leonidas Tapiero-Ortiz[1]; Nathali López-Cardona[1]; Alejandra Guevara-Castro[1]; Sandra Milena Rodríguez-Triana[2]; Edisson Chavarro-Mesa[3]; Jorge Evelio Ángel-Diaz[4]

[1]Corporación Colombiana de Investigación Agropecuaria – AGROSAVIA, C.I. La Libertad. Km 17 vía Puerto López, Villavicencio, 500008, Colombia; [2]SEMILLANO, Cl. 5b 22-36, Villavicencio, 500004, Colombia. [3]Universidad Tecnológica de Bolívar, Facultad de Ciencias Básicas. Km 1 vía Turbaco, Cartagena de Indias, 130012, Colombia; [4]Instituto Colombiano Agropecuario – ICA. Carrera 68A N° 24B-10 Edificio Plaza Claro-Torre B Bogotá D.C. 111210, Colombia.

Autor para correspondência: Anibal Leonidas Tapiero (atapiero@unal.edu.co)

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ABSTRACT

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Soybean crops grown in the plains of eastern Colombia have been affected by the incidence of rust. This disease, still officially regarded as American soybean rust (caused by *Phakopsora meibomiae*) by the Colombian Department of Agriculture (ICA), causes serious damage to soybean growing areas. Symptoms of rust, such as reddish-brown lesions, have been observed since 2004 in the upper half of plants during vegetative stages. In 2005 and 2018, infected leaf tissues and uredinospores were collected from an experimental area and from commercial soybean fields. Once morphological identification and Koch's postulates confirmed the presence of *Phakopsora* spp., molecular characterization was performed to identify the pathogen associated with the disease to species level. This was based on standard procedures using internal transcribed spacer (ITS) regions of rDNA and PCR amplification with specific primers for *Phakopsora meibomiae* and *P. pachyrhizi*. The obtained sequences were BLASTed against GenBank/NCBI data bank. Results indicated that *P. pachyrhizi* Sydow is in fact the causative agent of soybean rust in Colombia, considering the samples collected in 2005 and 2018. The ITS-rDNA sequences of *P. pachyrhizi* were deposited at GenBank under the accession numbers MK933723 to MK933731. This finding was reported to ICA, so that they could officially update the phytosanitary status of this soybean pathogen in Colombia.

Keywords: *Glycine max*, *Phakopsora* spp. phenotyping, phylogenetic analyses, maximum likelihood method, nucleotide sequencing, ITS region.

RESUMO

Tapiero-Ortiz, A.L.; López-Cardona, N.; Guevara-Castro, A.; Rodríguez-Triana, S.M.; Chavarro-Mesa, E.; Ángel-Diaz, J.E. *Phakopsora pachyrhizi* é o agente causal da ferrugem da soja na Colômbia. ***Summa Phytopathologica,*** v.47, n.3, p.149-156, 2021.

As culturas de soja produzidas nas planícies do leste da Colômbia vem sendo afetadas pela incidência de ferrugem. A doença, ainda considerada oficialmente, pelo Departamento de Agricultura da Colômbia (ICA) como ferrugem americana (ocasionada pelo fungo *Phakopsora meibomiae*), causa sérios danos à produção nas áreas de cultivo da soja. Os sintomas têm sido observados desde 2004 na metade superior das plantas durante os estágios vegetativos, como lesões marrom-avermelhadas. Em 2005 e 2018, tecidos foliares infectados e uredinosporos foram coletados em uma área experimental e cultivos comerciais dessa cultura. Uma vez que a identificação morfológica e os postulados de Koch confirmaram a presença de *Phakopsora* spp., foi realizada uma caracterização molecular para se identificar, a nível de espécie, o patógeno associado à ferrugem da soja. Estes foram baseados em procedimentos padrão com espaçadores de transcrição internos (ITS) do rDNA e amplificação por PCR com primers específicos para *P. meibomiae* e *P. pachyrhizi*. As sequências obtidas foram verificados por meio do Blast no site do GenBank/NCBI. Os resultados indicaram que *Phakopsora pachyrhizi* Sydow é, de fato, o agente causal da ferrugem da soja na Colômbia, considerando-se as amostras coletadas em 2005 e 2018. As sequências do ITS-rDNA de *P. pachyrhizi* foram depositadas com os números de acesso do GenBank MK933723 até MK933731. A informação foi repassada ao ICA, para que atualize oficialmente a situação fitossanitária desse patógeno da soja na Colombia.

Palavras-chave: *Glycine max*, fenotipagem de *Phakopsora* spp., análises filogenéticas, método de máxima verossimilhança, sequenciamento de nucleotídeos, região ITS.

Since 2004, rust has caused severe damages to more than 22,500 ha of Colombian soybean fields. Although there is a certain broad array of lands suitable for growing soybean in Colombia, the crop is currently mostly cultivated in the eastern plains (11). Only small scattered soybean crops remain on the best soils available at Valle del Cauca, where soybean cultivation once flourished. Besides, the Colombian soybean area shrank to 22,500 ha from more than 36,000 ha grown until 2004 (4) due to production constrains, such as the cost devoted to control rust. Flat or slightly sloping savannas, in "Orinoquia" region, characterize the land on the eastern plains. The soils there are poor, contain low organic material, are very acid (pH around 4.6 – 5.4) and present high aluminum levels. Climate is typically neo-tropical with a bi-modal rainfall pattern (mean 2,800 mm/yr) and a major dry season from December to March. The environment (high humidity and air

temperatures above 24ºC) across the crop season is highly conducive to the disease.

Soybean rust typically shows reddish-brown and tan-colored lesions (12), starting at vegetative stages (V3 – V4), from the lower canopy up. The disease rapidly progresses upward on the plant, until all leaves present some level of the disease or become completely defoliated, under favorable conditions. Pustule-like structures called uredinia are soon observed developing on the affected tissues. Uredinia harm the leaf tissue, causing drops in soybean yields of 30% to 80% (17).

Two closely related species of *Phakopsora* cause soybean rust (15). *Phakopsora pachyrhizi* Sydow was considered restricted to Australasia, Japan, the Philippines, and Taiwan until 1990. In South America, Central America and the Caribbean, only *P. meibomiae* (Arthur) Arthur was reported causing rust until 2000. However, in 1992, *P. pachyrhizi* was first reported causing soybean rust in Hawaii (15); similarly, in 2002, it was first reported affecting soybean in Brazil (13), Paraguay and Argentina (16).

Despite several reports on the supposed occurrence of *P. pachyrhizi* in Colombia (3, 6, 7, 14, 23), soybean rust is still officially associated with *P. meibomiae* by the Colombian Department of Agriculture, ICA (8). This is based only on morphological characteristics of the telia of *Phakopsora* spp. (2, 15). ICA's official statement about the *Phakopsora* species associated with soybean rust does not recognize as valid the phenotyping of two isolates (CO 04-1C and CO 04-1D) analyzed and stored in the USDA-ARS Foreign Disease-Weed Science Research Unit (FDWSRU) at Ft. Detrick, Frederick, MD, which were shipped independently by a local farmer from Caicedonia, Valle del Cauca (4.3433333' N, 75.8136111' W). Therefore, in the absence of a scientific study based on a systematic sampling, the official status of the soybean rust pathogen species remains as previously designated by ICA.

A number of different molecular markers have been implemented for *Phakopsora* population analyses and used elsewhere (5, 18, 21, 22, 23). These include both classical and real-time PCR assays.

Nucleotide sequences of the ITS1 and ITS2 regions of *P. pachyrhizi* and *P. meibomiae* isolates, which ranged in size from 196 to 199 nucleotides for *P. pachyrhizi* isolates and 218 nucleotides for *P. meibomiae* isolates, were gathered, showing more than 98.0% sequence identity between isolates (5). A comparison between *P. pachyrhizi* and *P. meibomiae* ITS1 regions revealed nucleotide differences representing 77.5% sequence identity between the two *Phakopsora* species. ITS2 region ranged from 199 to 206 nucleotides for *P. pachyrhizi* and from 203 to 205 nucleotides for *P. meibomiae*. Within species, identity was over 95.0% among sequences from *P. pachyrhizi* isolates and higher than 99.0% among sequences from *P. meibomiae* isolates. Comparisons between ITS2 regions of *P. pachyrhizi* and *P. meibomiae* revealed approximately 68.5% nucleotide sequence identity between these species. Ten nucleotide differences or gaps were found among *P. pachyrhizi* isolates (resulting in more than 95.0% identity), while only two additional nucleotides were found for *P. meibomiae* isolates (resulting in more than 99.0% identity). Frederick et al. (5) deposited the nucleotide sequences of ITS1 - 5.8S - ITS2 regions from *P. pachyrhizi* and *P. meibomiae* as GenBank Accession Numbers AF333488 to AF333502.

Since nucleotide sequence comparisons revealed significant divergence between species, Frederick et al. (5) selected sequence sites to design PCR primers that were useful for distinguishing between *Phakopsora* species. The PCR primers Ppa1, Ppa2, Ppa3 and Ppa4 were specifically designed for *P. pachyrhizi*, while Pme1 and Pme2 targeted the specific amplification of *P. meibomiae*. Furthermore, primers Ppm1 and Ppm2 were designed with the aim of discriminating between specific sites in the 5.8S rDNA region, which is conserved in these two species.

Considering the epidemiological behavior of soybean rust in the field, particularly its rapid spread at early vegetative stages and the severe damage caused to leaf tissues, the present study tested new approaches to resolving a relevant etiological issue about the actual *Phakopsora* species causing soybean rust in Colombia. The study was conducted based on ITS-rDNA molecular markers, which allow specific identification and discrimination between *P. meibomiae* and *P. pachyrhizi*.

MATERIALS AND METHODS

The soybean rust pathogen samples used for this study were obtained at two stages: in 2005, after rust lesions were observed in soybean trap plots at AGROSAVIA La Libertad Experimental Center, and in 2018, after widespread occurrence of the disease on commercial fields was documented. The collected source cultivars were 'Williams', 'Soyica P34', 'Corpoica Orinoquia 3', and 'Corpoica La Libertad 4' for the first

Table 1. Sample source and origin for the 2018 sampling of rust-like isolates.

Municipality	County	Latitude	Longitude	Altitude	Cultivar
Puerto Gaitán	Alto Neblinas	4.32436	-72.02012	198	Panorama 29i
Puerto López	Remolinos	4.21191	-72.64508	204	Panorama 29i
Puerto López	Yurimena	4.18436	-72.59759	214	Panorama 29i
Puerto López	Remolinos	4.20729	-72.66458	202	Panorama 29i
Puerto Gaitán	Alto Neblinas	4.32436	-72.02012	198	Achagua 8
Puerto López	Yurimena	4.18436	-72.59759	214	Achagua 8
Villavicencio	La Reforma	4.03286	-73.28107	355	Sloan
Villavicencio	Pompeya Bajo	4.00078	-73.37435	243	Soy - SK7
Puerto Gaitán	La Cristalina	4.27685	-71.7261	241	BRS- Serena

sampling, besides 'Panorama 29', 'Corpoica Achagua 8', 'Sloan', 'Soy Sk-7', and 'BRS Serena' for the second sampling (Table 1).

In 2005, pale yellow-brown to colorless urediniospore samples were collected from 2–5mm-diameter individual lesions on the lower leaf surface of plants (Figure 1) by using a pump-driven spore collector (Cyclone surface sampler, Burkard Manufacturing Co. Ltd. Rickmansworth, UK). The spores were then kept dry on Eppendorf (1.5ml) tubes until lab processing. For the 2018 sampling, infected leaves were collected from the field; then, tissue sections showing erumpent lesions were placed on Petri dishes with humidified filter paper. Afterwards, the morphometric characters of collected spores were confirmed based on the procedures reported by Ono et al. (15) for *Phakopsora* spp.; pathogenicity tests were also performed.

The first set of spore samples (2005 sampling) was placed on Petri dishes containing sterile water for germination and subsequent DNA extraction. Uredinospores from the second sampling (2018 sampling) were kept in Eppendorf tubes added of 1ml sterile water on glycerin at -20ºC, until processing. PCR amplification, sequencing and GenBank/NCBI database search procedures were similar for both batches of samples. Specimen collection was conducted with the permit granted to AGROSAVIA by the Colombian National Environmental Licensing Authority (ANLA's Resolution No. 1466 of December 03, 2014).

DNA extraction was performed from both mycelial growth on sterile water (Figure 2) and urediniospore lysis at 95ºC for 8 min using a thermocycler (PTC- 100 MJ Research, Inc., Watertown, MA, USA). DNA extraction followed the PowerSoil DNA Isolation Kit protocol. DNA concentrations were adjusted to over 200 ng/µL, after the initial DNA concentration of samples was determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). PCR amplification was performed on 25µL final volumes of 9 DNA samples composed of the following aliquots: 12.5 µL Go Taq Green Master Mix; 2 µL each primer (forward and reverse, 10 µM); 5.5 µL sterile water; and 3 µL extracted DNA samples.

Nucleotide sequencing of the internal transcribed spacer (ITS) region (2018 sampling only).

The ITS regions from *P. pachyrhizi* and *P. meibomiae* isolates were PCR-amplified from nine selected *Phakopsora* spp. isolates. This was done with the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'), according to the protocols of Udayanga et al. (20). Primers ITS4 and ITS5 were initially denaturized at 96°C for 2 min (35 cycles), followed by denaturation at 96°C for 1 min, alignment at 55°C for 1 min,

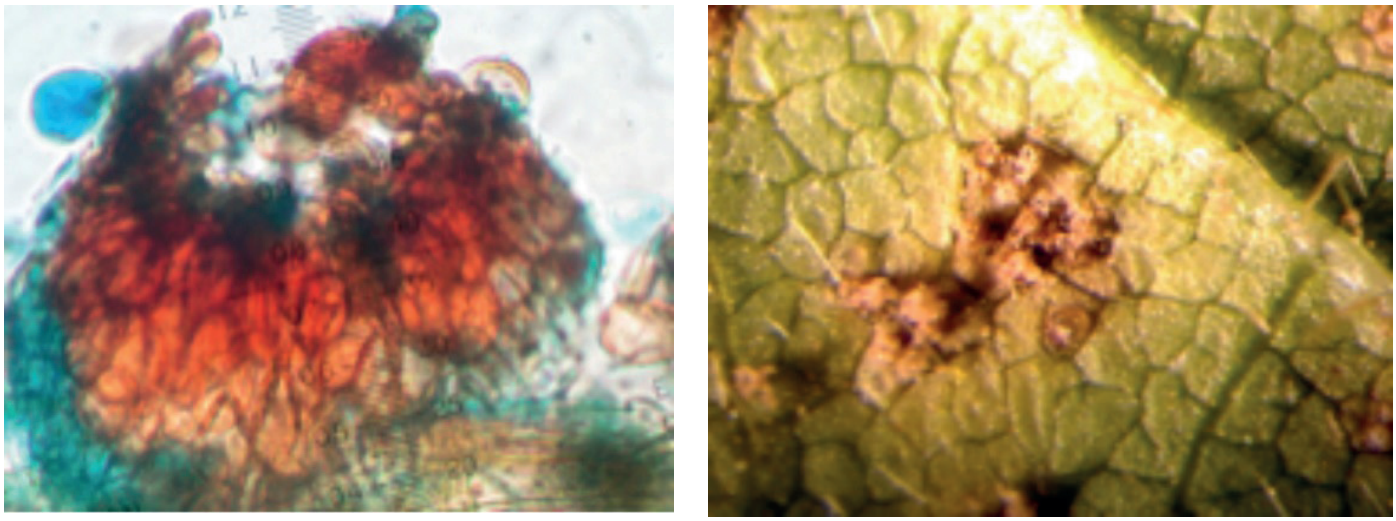

Figure 1. Sub-epidermal (left) and erumpent pale yellow-brown to colorless uredia (right, 0.4mm diameter).

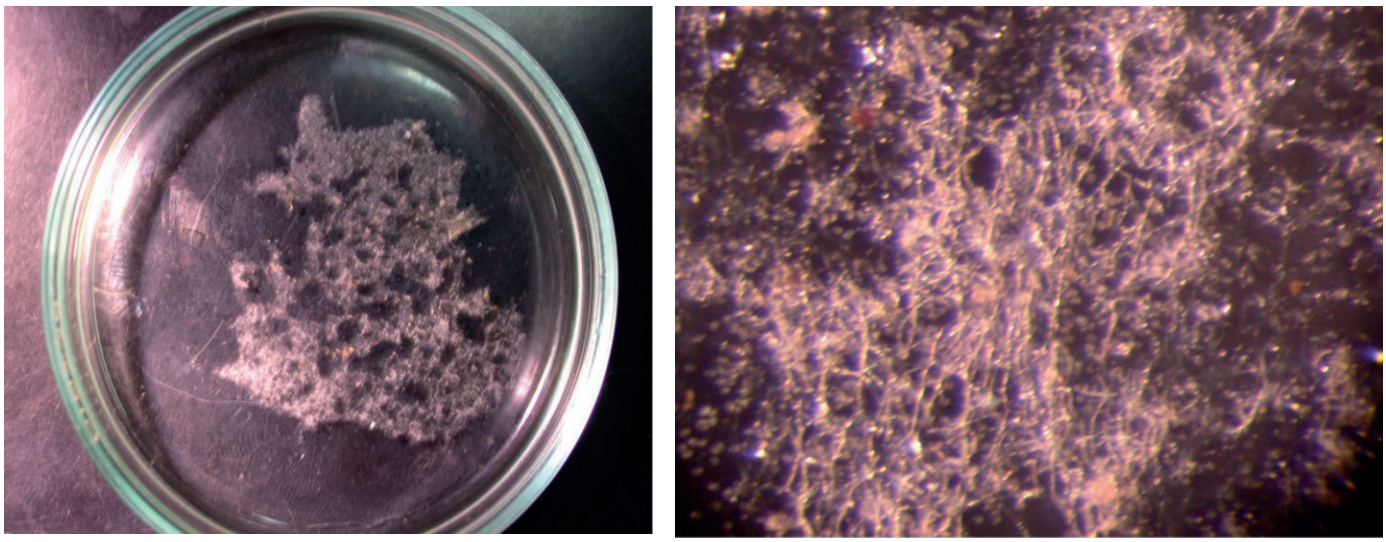

Figure 2. Mycelial growth on sterile water.

and extension at 72°C for 2 min. Finally, extension was programmed at 72°C for 10 min.

Phylogenetic analysis by Maximum Composite Likelihood method.

The phylogenetic distances of the isolates' sequences and phylogenetic trees were inferred according to the Maximum Composite Likelihood (MCL) method and Kimura 2-parameter model (9). The phylogenetic tree with the highest log likelihood (-1218.66) was built with the percentage of trees in which the associated taxa clustered together (next to the branches). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated based on the MCL approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured by the number of substitutions per site and bootstrap value (confidence) labels. The performed analysis involved 32 nucleotide sequences. The included codon positions were $1^{st} + 2^{nd} + 3^{rd}$ + noncoding. Phylogenetic analyses were conducted in MEGA X (10).

Identification of *Phakopsora* species with specific primers.

Four pairs of oligonucleotide PCR primers selected for their specificity to *P. pachyrhizi* (Ppa1/Ppa2, Ppa3/Ppa4, Ppm1/Ppa2, and Ppm1/Ppa4) were used to discriminate *P. pachyrhizi* from the isolates sampled in 2005. Only Ppa1/Ppa2 oligonucleotide set was used for the isolates collected from commercial crops in 2018. The specific oligonucleotide PCR primers (Pme1/Pme2 and Ppm1/Pme2), designed to amplify products from *P. meibomiae* but not from *P. pachyrhizi*, were used to discriminate the causative organism from the 2005 sampling. Ppm1/Pme2 oligonucleotide primer set that amplified a 79-bp PCR product from both *P. pachyrhizi* isolate TW72-1 and *P. meibomiae* was used for the isolates collected in 2018.

Oligonucleotide primers were Ppm1 (5'-GCAGAATTCAGTGAATCATCAAG-3') and Pme2 (5'-GCACTCAAAATCCAACATGC-3') for *P. meibomiae*; Ppm1 (5'-GCACTCAAAATCCAACATGC-3') and Ppa2 (5'-GCAACACTCAAAATCCAACAAT-3'); Ppa3 (5'-CCCATTTAATTGGCTCATTG-3') and Ppa4 (5'-TCAAAATCCAACAATTTCCC-3') for *P. pachyrhizi*. PCR conditions were as follows: Initial denaturalization at 95°C for 3 min; followed by denaturalization at 95°C for 1 min (35 cycles); annealing at 55°C for 30s; extension at 72°C for 1 min, and final extension at 72°C for 10 min. The amplified products were observed on 2% agarose gel (5).

Analysis and editing of sequences from the ITS region and from the cloning of isolates by specific primers were performed with Geneious v 8.1.9 software. The consensus sequences generated from the assembly of the sequenced reactions were analyzed by running the Basic Local Alignment Search Tool (BLAST) at NCBI database to determine their homology to nucleotide sequences (Table 2).

RESULTS AND DISCUSSION

Comparison of *P. pachyrhizi* and *P. meibomiae* sequenced ITS regions

Nucleotide alignment from amplified sequences of the Internal transcribed spacer (ITS) conserved regions: ITS4 (5-`TCCTCCGCTTATTGATATGC'-3) and ITS5 (5-`GGAAGTAAAAGTCGTAACAAGG'-3 using BLAST searching engine at NCBI libraries indicated 98%–100% agreement with *P. pachyrhizi* for the 9 sequenced Colombian rDNAs (Table 3). Molecular weights (625–880bp) were within the expected size frame (641–668bp) of the ITS's linear DNA reported by Frederick et al. (5), which were deposited as GenBank Accession Number AF333488 to AF333502.

Evolutionary analysis by the Maximum Likelihood method

The phylogenetic tree built after MCL showed 3 well-defined clades (Figure 3). Distances, related to 0.1 substitutions per site scale, show that the majority (8 out of 9) of Colombian isolates' sequences clustered at clade I (bootstrap 90), together with the isolate sequences reported by Frederick's et al. (5), which were obtained based on a final dataset of 397 positions. One isolate (MK933726) clustered with several USA isolates at clade III (bootstrap 99), and no sequence was aligned to sequences at clade II, which groups *P. meibomiae* isolates from Puerto Rico and Panama (bootstrap 99). Clade I shows a somehow well-defined subset of isolates organized in three sub-clades, while clades II and III are unanimously single. Topology distance between clades II and I indicates a clear separation of *P. pachyrhizi* from *P. meibomiae*. Isolate MK933726, at clade III, joined a set of highly predominant A5 and T5 genotypes, found at 23.0% and 55.7% frequencies, respectively, in samples of rain collected across the USA between 2005 and 2006 (1). All but one isolate (EU436718) belongs to genotypes T5, grouped together at clade III. T genotypes were named according to one single nucleotide polymorphism in thymine substitution at the 3´-end of ITS1 region plus a number indicating the variation in TA repeats. On the other hand, A genotypes were named based on one adenine substitution at the

Table 2. Sequence and BLAST output of the ITS4 and ITS5 amplified regions of an isolate from the cultivar 'Soy - SK7', Pompeya Bajo County, Villavicencio, Colombia.

Species: *Phakopsora pachyrhizi*; identity: 100%
Sequence:
AGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATAAAAAGCTAAAGAGTGCACTTTATT-GTGGCTCAAAACTAAACTTTTTAATAAACCCATTTAATTGGCTCATTGATTGATAAGATCTTTGGGCAATGGTAGCTTT-GAAAAAAGCTGCAACCCACCTATTAATCATAATCTTTTTTTTTTAACTCAAAGTCAAATAGAATGTTTTATAAATTTAAATA-TATATATAACTTTTAACAATGGATCTCTAGGCTCTCATATCGATGAAGAACACAGTGAAATGTGATAATTAATGTGAATTG-CAGAATTCAGTGAATCATCAAGTTTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCAT-GAAATCTTCTCAACATTATTTCTTTTTAAAGGAAATTGTTGGATTTTGAGTGTTGCTGTTGCTTTTTTTGCAGCTCACTTTAAA-TAAATAAATATATATAAGTTTCAGTATATTTTGATGTAATAATAAAATCATTTCATCAAAAAAATAAATATATGTGAGATT-TATTATAACATTAATTGAATGTAATTTTTTTTTTTAAGACCTCAAATCAGGTGAGACTACCCACTGAACTTAAGCATATCAA

Table 3. Colombian isolates' DNAs for 2018 sampling, ITS GenBank accessions and sequence identity with *Phakopsora pachyrhizi* from a BLAST search.

Isolate	County	.Accession No	(Identity (%)/size (bp
SUB5621774 **009**_P._pachyrhizi_COLOMBIA	La Cristalina	MK933723	99/626
SUB5621774 **008**_P._pachyrhizi_COLOMBIA	Pompeya	MK933724	100/648
SUB5621774 **007**_P._pachyrhizi_COLOMBIA	La Reforma [1]	MK933725	99/654
SUB5621774 **006**_P._pachyrhizi_COLOMBIA	Yurimena	MK933726	100/880
SUB5621774 **005**_P._pachyrhizi_COLOMBIA	Alto Neblinas	MK933727	100/641
SUB5621774 **004**_P._pachyrhizi_COLOMBIA	Remolinos	MK933728	99/661
SUB5621774 **003**_P._pachyrhizi_COLOMBIA	Yurimena	MK933729	99/654
SUB5621774 **002**_P._pachyrhizi_COLOMBIA	Remolinos	MK933730	99/657
SUB5621774 **001**_P._pachyrhizi_COLOMBIA	Alto Neblinas	MK933731	98/656

1: AGROSAVIA La Libertad Research Station, geographical coordinates: 4.0614402'N 73.5031149'W; 338.46 MAMSL

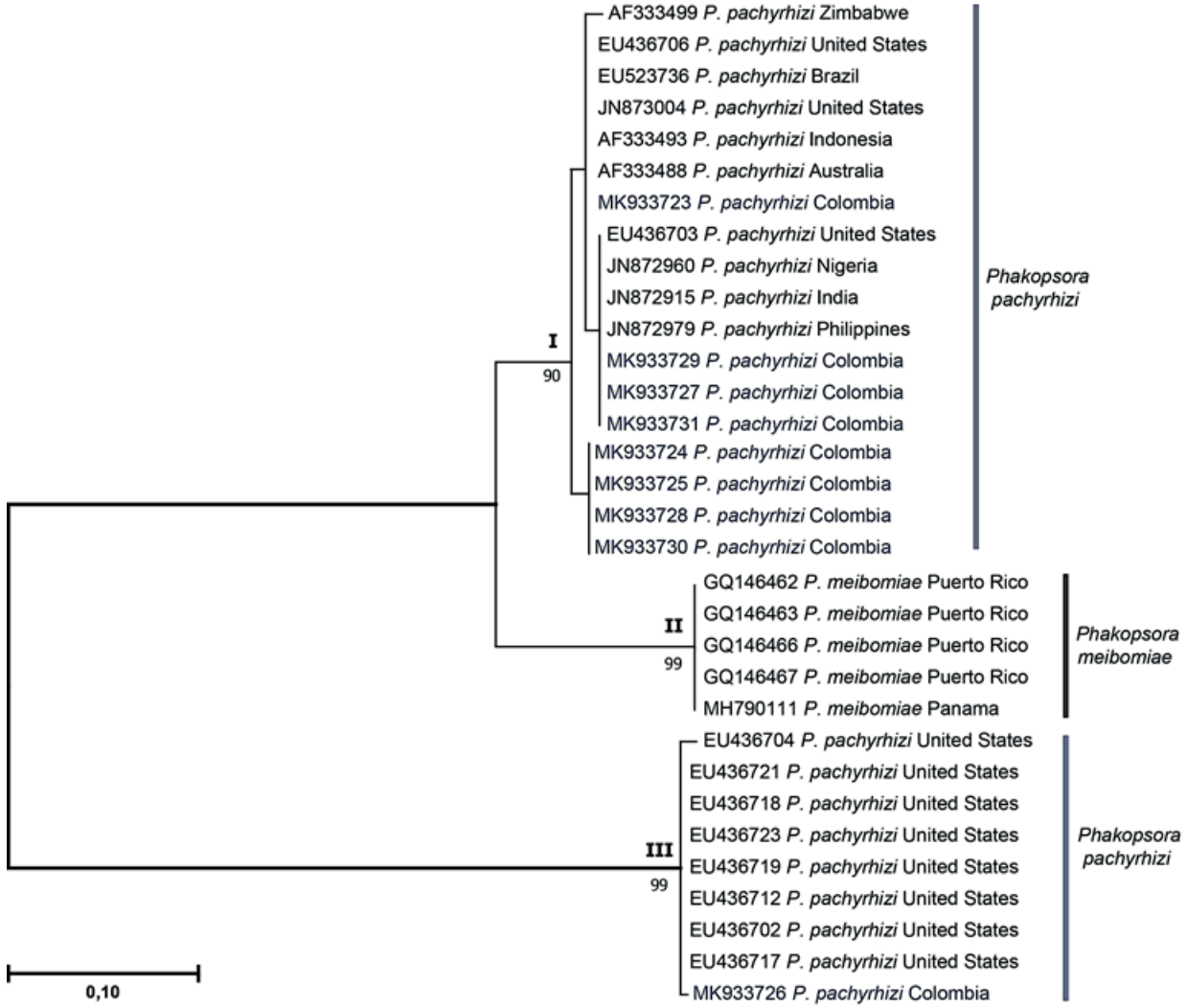

Figure 3. Maximum Likelihood sequence distance trees output from Colombian *Phakopsora pachyrhizi* DNA's ITS samples related to Frederick et al. (4) accessions. Bootstrap values (after 10000 replicates) are indicated at the nodes.

3´-end of ITS1 plus the same number related to variations in TA repeats.

Identification of *Phakopsora* species with specific primers

All 9 PCR-amplified DNA samples of isolates collected in 2018 and the 5 samples obtained in 2005 had negative results for *P. meibomiae* (no fragments amplified) using specific nucleotide primers Ppm1/Pme2 and Pme1/Pme2. A positive fragment (136bp) was observed after amplification with oligonucleotide primers specific to *P. pachyrhizi*, Ppm1/Ppa4, in 2018 sampling (Figure 4). DNA amplifications from 2005 sampling were all positive for *P. pachirhizi*. The size of nucleotide fragments with oligonucleotide primers Ppm1/Ppa2 was 141bp, while that with Ppm1/Ppa4 was 136bp (Figure 5). On the other hand, Ppa1/Ppa2 returned a 332bp fragment, and Ppa3/Ppa4 showed 347bp. Both results confirmed the identity of the isolate sequences as belonging to *P. pachyrhizi* (Figure 6).

These results indicate that the amplified PCR products from 2005 and 2018 samplings, which ranged in size from 136 to 367bp with specific oligonucleotide primers (Ppm1/Ppa2; Ppm1/Ppa4, and Ppa1/Ppa2), corroborate the DNA amplification sizes reported for *P. pachyrhizi* isolates by Frederick et al. (5). The same was stated for the isolates CO 04-1C and CO 04-1D by Zhang et al. (22) and all Colombian isolates grouped in clades with *P. pachyrhizi* isolates from several countries. Moreover, as specific primers Ppm1/Pme2 and Pme1/Pme2 yielded no product from *P. meibomiae*, it is straightforwardly confirmed that *P. pachyrhizi* is the causative agent of soybean rust in Colombia and, to our understanding, this pathogen has been present in Colombian soybean fields since at least 2004.

New insights into the pathotyping of microorganisms subject to benzimidazole pressure, such as *P. pachirhizi,* have been proposed (19). Thus, *Phakopsora* species would be better discriminated by means of sequencing nucleotides of β-tubulin gene (Pp tubB), associated with benzimidazole resistance, as reported for the closely related model organisms *Aspergillus nidulans* and *Neurospora crassa*. However, the usefulness of Pp tubB for phylogeographic discrimination of *P. pachyrhizi* at seven commercial soybean fields in Brazil proved to be poorly adequate for phylogenetic analysis and should be disregarded, in contrast with reports of high level of sequence identity between Pp TUB and β-tubulins of the previously mentioned model organisms. Nevertheless, there is a still clear relationship between amino acid

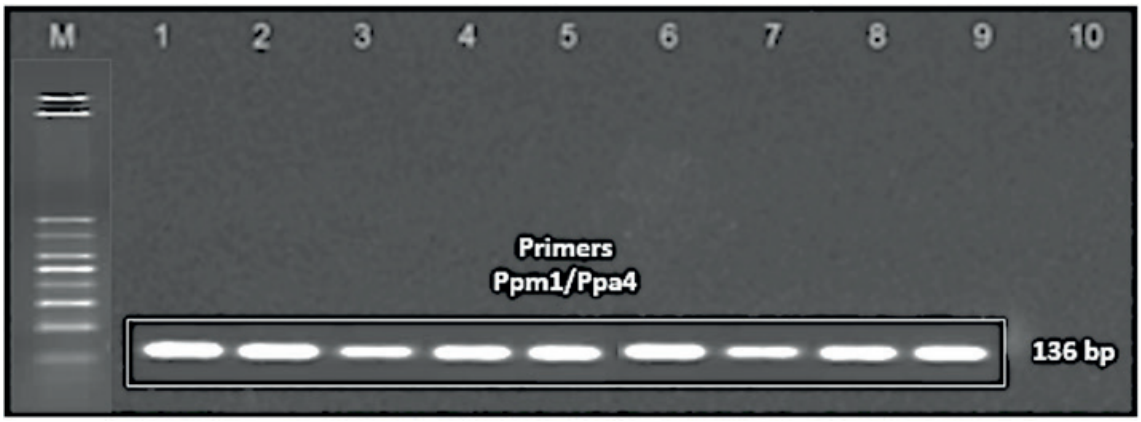

Figure 4. Agarose gel (1.8% w/v) with DNA amplification products of specific oligonucleotide primers (Ppm1/Ppa4) for *Phakopsora pachyrhizi*. Lanes M: DNA Ladder (100 bp). Lanes 1-9: DNA from 2018 samples. Lane 10: negative control. (Sybr safe stain).

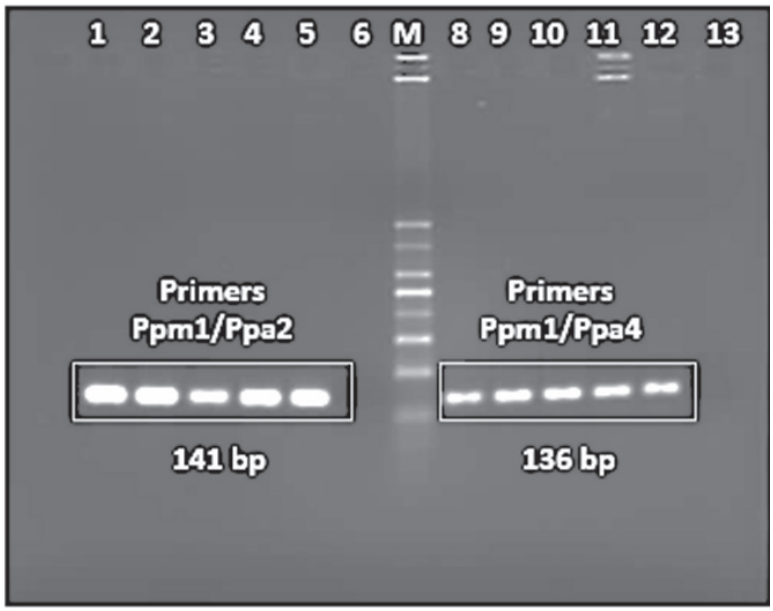

Figure 5. Agarose gel (1.8% w/v) with DNA amplification products of specific primers (Ppm1/Ppa2) and (Ppm1/Ppa4) for *Phakopsora pachyrhizi*. Lane M: DNA Ladder (100 bp). Lanes 2-5 and 8-11: DNA sampled from infected cultivars 'Williams', 'Soyica P34', 'Corpoica Orinoquia 3' and 'Corpoica La Libertad 4' in 2005. Lanes 5 and 12: positive reaction control. Lanes 6 and 13: negative control.

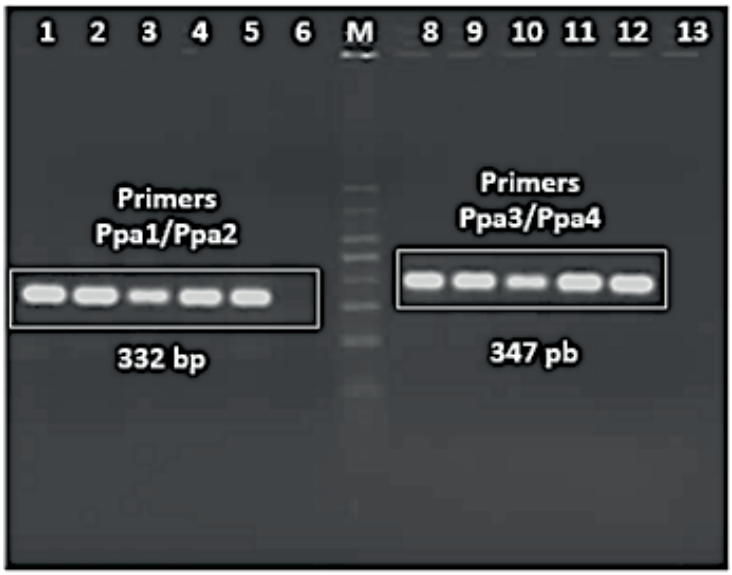

Figure 6. Agarose gel (1.8% w/v) with DNA amplification products of specific primers (Ppa1/Ppa2) and (Ppa3/Ppa4) for *Phakopsora pachyrhizi*. Lane M: DNA Ladder (100 bp). Lanes 1-4 and 8-11: DNA sampled from infected cultivars 'Williams', 'Soyica P34', 'Corpoica Orinoquia 3' and 'Corpoica La Libertad 4' in 2005. Lanes 5 and 12: positive reaction control. Lanes 6 and 13: negative control.

substitutions at given sites and benzimidazole resistance. The presence of only four polymorphic sites after Pp tubB testing led to the conclusion that the amino acid substitutions in β-tubulin were absent in *P. pachyrhizi* sequences. Therefore, identification of *Phakopsora* species using specific primers and sequencing of ITS regions are still regarded as the most useful and powerful approach to discriminate between *P. pachyrhizi* and *P. meibomiae*.

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The present study deals with newly approaches to type *Phakopsora* populations collected from Colombian soybean fields at different times. Using ITSs and specific primers as molecular markers, the current study demonstrated that the soybean rust pathogen, *P. pachyrhizi,* is present since the beginning of the XXI Century. Thus, the status of rust pathogen must be officially given to *P. pachyrhizi* instead of *P. meibomiae* by the phytosanitary authorities. Phenotyping *Phakopsora* populations with molecular markers allows better understanding the disease and more adequate epidemiological surveillance of the pathogen. This will provide more useful information to improve the efficiency of the disease management strategies, such as the use of resistant varieties and chemical control.

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