e-Xtra*

Variation in the Internal Transcribed Spacer Region of *Phakopsora pachyrhizi* and Implications for Molecular Diagnostic Assays

Tomás Allen Rush,[1,2] Jacob Golan,[3] Alistair McTaggart,[4] Cade Kane,[5] R. W. Schneider,[1] and M. Catherine Aime[5,†]

[1] Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, U.S.A.
[2] Department of Plant Pathology, University of Wisconsin, Madison, WI 53706, U.S.A.
[3] Departments of Botany and Bacteriology, University of Wisconsin, Madison, WI 53706, U.S.A.
[4] Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Ecosciences Precinct, Brisbane, Queensland 4001, Australia
[5] Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, U.S.A.

Abstract

Phakopsora pachyrhizi, the causal agent of soybean rust (SBR), is a global threat to soybean production. Since the discovery of SBR in the continental United States, quantitative polymerase chain reaction assays based on the internal transcribed spacer (ITS) ribosomal DNA locus were established for its rapid detection. However, insufficient data were initially available to test assays against factors that could give rise to misidentification. This study aimed to reevaluate current assays for (i) the potential for false-positive detection caused by nontarget *Phakopsora* species and (ii) the potential for false-negative detection caused by intraspecific variation within the ITS locus of *P. pachyrhizi.* A large amount of intraspecific and intragenomic variation in ITS was detected, including the presence of polymorphic ITS copies within single leaf samples and within single rust sori. The diagnostic assays were not affected by polymorphisms in the ITS region; however, current assays are at risk of false positives when screened against other species of *Phakopsora*. This study raises caveats to the use of multicopy genes (e.g., ITS) in single-gene detection assays and discusses the pitfalls of inferences concerning the aerobiological pathways of disease spread made in the absence of an evaluation of intragenomic ITS heterogeneity.

Keywords: disease forecasting, DNA barcoding, IPM-PIPE, Pucciniales, Uredinales

Phakopsora pachyrhizi Syd. & P. Syd., the causal agent of soybean rust (SBR), is one of several species of rust fungi that cause disease on soybean [*Glycine max* (L.) Merr] (Ono et al. 1992). In some countries, SBR has reportedly caused losses of >50% of soybean yield (Bonde et al. 2006; Fletcher et al. 2010; Kelly et al. 2015; Sikora et al. 2014), and the disease caused profit losses of up to $2 billion in Brazil in 2003 (Yorinori et al. 2005).

Several detection methods were developed and implemented in response to outbreaks of SBR across the western hemisphere in the early 2000s (Allen et al. 2017; Isard et al. 2005, 2007, 2011; Kelly et al. 2015; Sikora et al. 2014; Young et al. 2011). Data obtained from traditional field and laboratory methods, based on morphology and symptomology, were uploaded to the Integrated Pest Management–Pest Information Platform for Extension and Education (2018) (IPM-PIPE) database, with which plant pathologists could monitor the spread and development of SBR. However, these data have limited value for preventing disease outbreaks and consequent yield losses. In an attempt to forecast SBR disease outbreaks, plant pathologists have developed a multifaceted approach involving sophisticated meteorological models (Esker et al. 2007; Isard et al. 2005), geographic information systems that track disease spread (Nelson et al. 1999; Plantegenest et al. 2007), and molecular identification of airborne urediniospores (Isard et al. 2011; Williams et al. 2001).

Frederick et al. (2002) developed conventional and real-time quantitative polymerase chain reaction (qPCR) assays based on the internal transcribed spacer (ITS) region of ribosomal DNA to detect and differentiate urediniospores of *P. pachyrhizi* and *P. meibomiae*, a closely related (and less severe) SBR fungal pathogen. Additional ITS-based detection assays were developed by Freire et al. (2008) to study the global population structure and migration routes of SBR and by Barnes et al. (2009) to detect and forecast *P. pachyrhizi* from spores collected in rainwash.

Barnes et al. (2009) tested their assay for false-positive results against *P. meibomiae* and nine species of *Puccinia*. However, 19 other species of *Phakopsora* are reported in the continental United States and Mexico (44 records total; Table 1), raising concern for the potential false-positive detection of *P. pachyrhizi*. In addition, at least 29 other species of *Phakopsora* are known from Central America (81 records) and 58 from South America (542 records) (Farr and Rossman 2018), where SBR continues to pose a threat to soybean. Many of these species of *Phakopsora* do not infect legumes but could possibly follow the same aerobiological pathways as *P. pachyrhizi* (Golan and Pringle 2017; Isard et al. 2005, 2007; Schumann and Leonard 2000), and it is unknown to what degree diagnostic primers of SBR amplify other species of *Phakopsora*.

The ITS region is widely accepted as the primary diagnostic barcode for fungal identification (Badotti et al. 2017; Schoch and Seifert 2012; Schoch et al. 2012). However, intraspecific and intragenomic dissimilarities among ITS copies have been observed in many fungi (Kiss 2012; Nilsson et al. 2008; Simon and Weiß 2008), especially those that are predominately asexual or polyploid (Eickbush and Eickbush 2007; Nei and Rooney 2005). Intragenomic ITS copies

†Corresponding author: M. C. Aime; maime@purdue.edu

T. A. Rush and J. Golan contributed equally to this work.

Funding: This research was supported by the United Soybean Board, U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service, Louisiana Board of Regents, National Science Foundation grants NSF-DBI-1458290 and NSF-DBI-1502887, and USDA-Hatch grant 1010662 to M. C. Aime.

*The *e*-Xtra logo stands for "electronic extra" and indicates that six supplementary figures and three supplementary tables are published online.

The author(s) declare no conflict of interest.

Accepted for publication 22 March 2019.

have been reported in several taxa of rust fungi, including *Chrysomyxa* species (Feau et al. 2011), *Coleosporium* species (McTaggart and Aime 2018), *Melampsora larici-populina* (Persoons et al. 2014), *Puccinia emaculata* (Uppalapati et al. 2013), and *Puccinia kuehnii* (Virtudazo et al. 2001).

Given that many fungal species house intraspecific and intragenomic variations in ITS, the specificity and reliability of diagnostic primers used in epidemiological tracking and diagnostics warrant more careful attention (Fig. 1). Therefore, one of the goals of this study was to assess the extent of intraspecific and intragenomic variation in ITS copies of *P. pachyrhizi* as well as the effect of such variation on the reliability of diagnostic assays. In establishing the degree of variability in ITS among SBR populations, and the extent of intragenomic variation within single urediniospores, our study highlights the need for greater caution in clinical protocols that rely on molecular identification from multicopy genetic loci.

Materials and Methods

Specimen collection and DNA extraction. A total of 29 specimens of *P. pachyrhizi* were collected from 21 individual soybean leaves, seven individual kudzu (*Pueraria montana* var. *lobata*) leaves, and one vial of detached spores from several soybean leaves (Table 2). All *P. pachyrhizi* specimens were collected between 2005 and 2017. *P. arthuriana*, *P. crotonis*, *P. meibomiae*, *P. gossypii*, *P. pachyrhizi*, *P. tecta*, and *Cerotelium fici* (syn: *P. nishidana*) were collected throughout the United States and supplemented with herbarium specimens (Table 2). *P. pachyrhizi* specimens were dried in a plant press, and voucher material was deposited in the Arthur Fungarium at Purdue University. Other specimens of *Phakopsora* and *C. fici* are vouchered in the U.S. National Fungus Collections and Louisiana State University Bernard Lowy Mycological Herbarium.

DNA of *P. arthuriana*, *P. crotonis*, *P. meibomiae*, *P. gossypii*, *P. pachyrhizi*, *P. tecta*, and *C. fici* were obtained from the U.S. National Fungus Collections (Beltsville, MD) (Aime 2006; Aime and Rossman, *unpublished data*; GenBank Accession Numbers are listed in Supplementary Tables S2 and S3). DNA of *P. pachyrhizi* and other species of *Phakopsora* was extracted from infected leaves using the MoBio UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). To avoid cross-contamination, each sample was carefully handled using new gloves and paper, and samples were extracted with sterile tweezers and scissors. Approximately 10 to 25 individual sori with spores were excised from a single leaf per sample. The quantity of DNA was measured using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE.).

Evaluation of previously developed *P. pachyrhizi*-specific PCR and qPCR assays. *Tests for false-positive reactions.* We evaluated assays developed by Frederick et al. (2002) and Barnes et al. (2009) for false-positive results with three replicated PCRs on

Table 1. Other species of *Phakopsora* found in the continental United States and Mexico

Species[a,b]	Syn. Genera[b]	Host Genera	Location	Reference
P. antiguensis	*Uredo antiguensis*	*Acalypha*	Mexico	Buritica (1999)
P. cherimoliae	*P. neocherimoliae*	*Annona*, *Jatropha*	Florida, Mexico	Alfieri et al. (1984), Anonymous (1960), Beenken (2014), Buritica (1999)
P. coca	None	*Erythroxylum*	Mexico	Buritica (1999)
P. compressa	*Angiospora*, *Physopella*, *Puccinia*	*Paspalum*	Mexico	Buritica (1999)
P. crotonis	*Bubakia*, *Melampsora*, *Milesia*, *Pucciniastrum*, *Schroeteriaster*, *Trichobasis*	*Croton*, *Crotonopsis*	Alaska, Arkansas, Arizona, California, Florida, Illinois, Indiana, Louisiana, Mississippi, Mexico, Texas, Nebraska, New Mexico, Tennessee	Alfieri et al. (1984), Buritica (1999), Cooke (1971), Cummins (1964, 1979), Gallegos and Cummins (1981), Gilbertson and McHenry (1969), McCain et al. (1990), Overholts (1938), Yohem et al. (1985)
P. cumminsiana	None	*Tripsacum*	Mexico	Buritica (1999)
P. desmium (syn: *P. gossypii*)	*Aecidium*, *Cerotelium*, *Kuehneola*, *Malupa*, *Uredo*	*Gossypium*	Florida	Alfieri et al. (1984), Anonymous (1960), Buritica (1999)
P. diehlii	None	*Aeschynomene*	Mexico	Gallegos and Cummins (1981)
P. jatrophicola (syn: *P. arthuriana*)	*Malupa*, *Uredo*	*Jatropha*	Florida, Mexico, Texas	Alfieri et al. (1984), Anonymous (1960), Buritica (1999), Gallegos and Cummins (1981), Hennen et al. (2005)
P. lenticularis	None	*Lasiacis*	Mexico	Buritica (1999)
P. mexicana	*Bubakia*, *Melampsora*, *Schroeteriaster*	*Croton*	Mexico, Texas	Buritica (1999), Gallegos and Cummins (1981)
P. mori	None	*Morus*	Florida, Mexico	Buritica (1999)
Cerotelium fici (syn: *P. nishidana*)	*Kuehneola*, *Malupa*, *Physopella*, *Uredo*	*Ficus*	Alabama	Buritica (1999)
P. nova	None	*Acalypha*	Mexico	Buritica (1999)
P. pallescens	*Angiospora*, *Dicaseoma*, *Physopella*, *Puccinia*, *Uredendo*, *Uredo*	*Euchlaena*, *Tripsacum*	Florida, Mexico	Buritica (1999)
P. tecta	*Physopella*	*Commelina*	Mexico, Texas	Buritica (1999), Gallegos and Cummins (1981)
P. zeae	*Angiopsora*, *Physopella*, *Uredendo*	*Zea*	Mexico	Buritica (1999)
P. zizyphi-vulgaris	None	*Ziziphus*	Florida	Alfieri et al. (1984), Anonymous (1960), Buritica (1999), Cummins (1962), Cummins and Stevenson (1956)

[a] Other species of *Phakopsora* that are found in the continental United States and Mexico that do not infect Vitaceae, based on the U.S. Department of Agriculture Agricultural Research Service Fungal Database (https://nt.ars-grin.gov/fungaldatabases/) (Farr and Rossman 2018). Records on species of *Phakopsora* that infect hosts in the family Vitaceae were excluded from Table 1 because they are polyphyletic compared with other species of *Phakopsora* (Aime 2006; Okane and Ono 2018; Ono et al. 1992).

[b] Validation of species names of *Phakopsora* found in the continental United States and Mexico based on those found in Index Fungorum (http://www.indexfungorum.org/).

DNA of *P. arthuriana*, *P. crotonis*, *P. meibomiae*, *P. gossypii*, *P. pachyrhizi*, *P. tecta*, and *C. fici. P. crotonis* was not tested with the Frederick et al. (2002) assay because of a limited amount of material.

We followed the conditions described by Frederick et al. (2002). qPCRs were amplified in a 25-µl reaction mixture with Ppm1 and Ppa2 as forward and reverse primers at a concentration of 0.5 µM, the FAM probe at a concentration of 300 nM, 1 µl of titanium Taq, 5 µl of titanium buffer, 0.5 µl of dNTP (2 mM), 1 µl of 20 ng of template genomic DNA, and sterile water up to 25 µl. qPCRs were run on an ABI Prism 7000 Sequence Real-Time PCR Detection System (Applied Biosystems Inc., Foster City, CA) with 7000 ABI System Software version 1.2.3.f.2 (Applied Biosystems Inc.). The cycling conditions were set at 94°C for 1 min, followed by 25 cycles of amplification (94°C for 15 s, 65°C for 15 s, 72°C for 15 s) and a final 6-min elongation step at 72°C.

The Barnes et al. (2009) assay has two stages: a preliminary PCR is performed, which is then diluted and used as the product for a nested qPCR. Preliminary PCRs were amplified in 25 µl with 0.5 µM of primers ITS1rustF10d and ITS1rustR3d, 1 µl of titanium Taq, 5 µl of titanium buffer, 0.5 µl of dNTPs (2 mM), 1 µl of 20 ng of template genomic DNA, and sterile water. The cycling conditions were 95°C for 2 min, followed by 20 cycles of amplification (95°C for 15 s, 60°C for 30 s, 72°C for 1 min), and a final 6-min elongation step at 72°C. We reduced the number of cycles from Barnes et al. (2009) to limit amplification of nonspecific products in later rounds of the PCR. The nested qPCR was amplified in 25 µl with 0.50 µM of primers ITS1rustF4a and Ppa2, 1 µl of the probe ITS1PhpFAM1, 1 µl of titanium Taq, 5 µl of titanium buffer, 0.5 µl of dNTPs (2 mM), 2 µl of preliminary PCR product (diluted to 1:1, 1:10, 1:100, or 1:1000), and sterile water. Barnes et al. (2009) only used a diluted concentration of 1:1,000. The cycling conditions were 95°C for 2 min, followed by 20 cycles of amplification (95°C for 15 s, 60°C for 30 s, 72°C for 30 s), and measurement of the quencher.

We measured the identity of each diagnostic primer for *P. pachyrhizi* to all species of *Phakopsora* with a publicly available ITS sequence. First, we mapped out all available specific primers and probes for *P. pachyrhizi* to the ITS locus (Fig. 2). Then primer sequences were aligned with MAFFT version 7 to 695 single-copy ITS sequences of *P. pachyrhizi* and 90 sequences from 19 other species of *Phakopsora* (Katoh et al. 2017; Kuraku et al. 2013). All alignments used default MAFFT settings except for the scoring matrix for nucleotide sequences, set to 1PAM/κ=2, as recommended for closely related DNA sequences. Alignments were then imported into R using the *seqinR* 3.4-5 package (Gouy et al. 1984), and the percent identity of a given primer to each of the 696 *P. pachyrhizi* as well as 90 other species of *Phakopsora* assessed with in-house scripts (Fig. 3).

Tests for false-negative reactions. We tested for false negatives caused by intraspecific variation in the ITS region for the assay developed by Barnes et al. (2009). The qPCR protocol outlined above was used to amplify different variants of the ITS region identified and cloned as described in the following section.

Comparison of ITS variants of *P. pachyrhizi* from a global collection. *Identifying variation of ITS in the United States.* The ITS region was amplified for 29 specimens of *P. pachyrhizi* using primers ITSPP3 and ITSPP5 with cycling conditions of 94°C for 5 min, 35 cycles of amplification (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min), followed by an extension period of 5 min at 72°C (Freire et al. 2008). The amplified ITS product from each sample of *P. pachyrhizi* was cleaned with an ethanol precipitation and ligated into a pGEM-T vector (Promega Corporation, Madison, WI) as instructed. Ligated PCR products were transformed in chemically competent *Escherichia coli* (ATCC 25922) cells and plated on Luria broth (LB) agar amended with ampicillin and incubated for 12 h. Colonies were selected from each LB agar plate based on colony size and lack of nearby satellite colonies, streaked onto a patch plate of LB agar with ampicillin, and stored at 4°C. Colonies were screened by PCR with the primers ITSPP3 and ITSPP5, and positive products were sent to Beckman Coulter Inc. (Morrisville, NC) for sequencing with ITSPP3 and ITSPP5 primers (Freire et al. 2008). Sequences were assembled using Sequencher version 4.1.4. (Gene Codes Corp., Ann Arbor, MI) and their identity was confirmed by BLAST searches in GenBank.

Determining whether variants of P. pachyrhizi *are monophyletic.* A phylogeny of *P. pachyrhizi* ITS variants was determined from a maximum likelihood search on 173 single-copy ITS sequences, including other species of *Phakopsora* and outgroups (Supplementary Fig. S1). Representative ITS sequences of eight *P. pachyrhizi* from GenBank and 108 from this study, 57 ITS sequences from 13 other

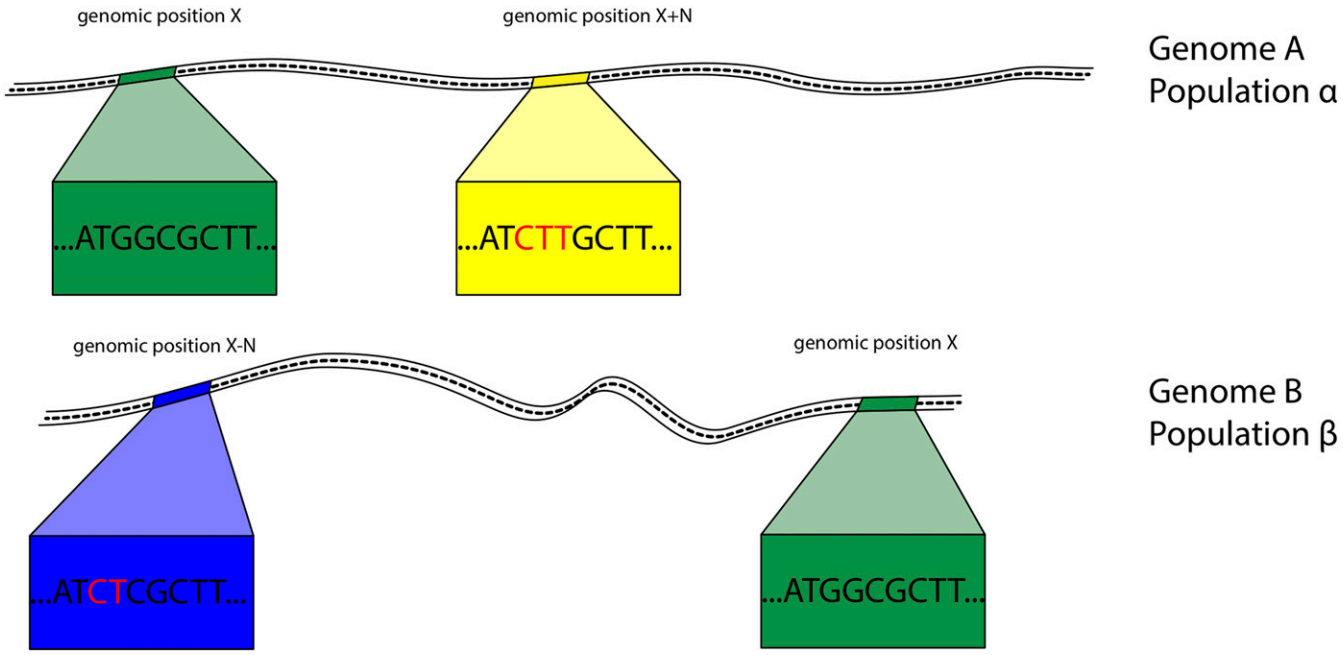


Fig. 1. An example of a polymorphic multicopy gene in two different genomes. Accurate population structure analyses must consider the relative position of each gene copy and compare copies from like genomic positions (X). A comparison of gene copies from dissimilar positions; for example, genomic position X+N to genomic position X would lead to erroneous conclusions regarding the differentiation between populations α and β in reference to the gene(s) under examination.

species of *Phakopsora*, and an outgroup, *Phragmidium tormentillae* (Genbank accession DQ354553; Aime 2006), were compiled from the National Center for Biotechnology Information (Supplementary Table S1) and aligned using MUSCLE in Mesquite version 3.4 (Maddison and Maddison 2008). Sequences were manually trimmed, and sequences for which an ITS subunit was missing were removed from the analysis. RAxML-HPC version 7.2.2 (Stamatakis 2014) searched for the best scoring likelihood tree with 100 rapid bootstraps (command -f a) using a GTRGAMMA model of evolution (Gatto et al. 2006).

Analysis of global ITS copy variation. ITS variation in *P. pachyrhizi* was evaluated across 211 dissimilar clonal, single-copy sequences from publicly available data sets and those obtained in the present study. This study analyzed cloned sequences of the ITS region generated from this study and 757 additional cloned sequences from other studies (864 total sequences; Barnes et al. 2009; de Jensen et al. 2013; Frederick et al. 2002; Freire et al. 2008, 2012; Jorge et al. 2015; Silva et al. 2008; Zhang et al. 2012). Sequences from samples collected in 15 different countries (Argentina, Australia, Brazil, China, Colombia, India, Nigeria, Paraguay, the Philippines, South Africa, Taiwan, Thailand, the United States, Vietnam, and Zimbabwe) were selected to encompass global ITS variation.

A single FASTA file of all 864 *P. pachyrhizi* ITS sequences was aligned using MAFFT version 7 (using default parameters except setting the scoring matrix to 1PAM/ κ=2 for closely related sequences) and imported into R version 3.4.0 using the *adegenet* and *poppr* packages (Jombart and Ahmed 2011; Kamvar et al. 2014). Only unique ITS copies from a given country were considered to approximate the behavior of sexual population and were employed in downstream analyses, for a total of 211 sequences (Grünwald and Hoheisel 2006; Grünwald et al. 2003; Milgroom 1996). To infer the number of genetic clusters across our global data set, and to evaluate the utility of single-copy ITS as a predictor of population structure in *P. pachyrhizi* (as proposed by Freire et al. 2008), the ΔK method was used to test the rate of change in the log-probability between successive cluster sizes among genotypes (where the number of clusters equals K; Goss et al. 2014; Supplementary Figs. S2 and S3). An unweighted pair group method with arithmetic mean dendrogram was then constructed from Nei's genetic distance between all pairs of the 211 genotypes (bootstrapping with 500 replicates)

Table 2. Locality, hosts, and date collected for species of *Phakopsora* that were used in this study

Species	Strain ID	State or Country[a]	County/ Municipality/ Parish/Province[a]	Coordinates[a]		Host	Date Collected[a]
				Latitude	Longitude		
P. crotonis	MCA 3227/ BPI 877960	Florida	St. Johns	29 86.598 N	81 26.731 W	Euphorbiaceae	10 September 2006
P. meibomiae	JRH 314/BPI 864114	Panama	Chiriquí	8 40.455 N	82 48.013 W	*Phaseolus multiflorus*	25 November 2004
P. meibomiae	JRH 456/BPI 863964	Puerto Rico	Guavate, Cayey	18 8.395 N	66 5.341 W	*Lablab purpureus*	5 December 2004
Cerotelium fici (syn: *P. nishidana*)	U9/BPI 842289	Puerto Rico	San Juan	NA	NA	*Fiscus* sp.	30 April 2003
P. pachyrhizi	TAR 103	Louisiana	Lafayette	29 58.820 N	091 45.686 W	*Pueraria montana* var. *lobata*	31 August 2009
P. pachyrhizi	TAR 106	Louisiana	NA	NA	NA	NA	NA
P. pachyrhizi	TAR 122	Louisiana	Caddo/Bossier	32 22.829 N	093 39.811 W	*Glycine max*	16 September 2009
P. pachyrhizi	TAR 129	Louisiana	Vermillion	30 00.514 N	092 21.131 W	*G. max*	18 September 2009
P. pachyrhizi	TAR 143	Louisiana	Pointe Coupe	30 30.647 N	091 30.573 W	*G. max*	29 September 2009
P. pachyrhizi	TAR 157	Louisiana	Allen	NA	NA	*G. max*	27 September 2009
P. pachyrhizi	TAR 158	Louisiana	Allen	30 33.873 N	092 40.302 W	*G. max*	25 September 2009
P. pachyrhizi	TAR 159	Louisiana	Calcasieu	30 03.585 N	093 10.750 W	*G. max*	28 September 2009
P. pachyrhizi	TAR 162	Louisiana	Union	32 55.921 N	092 37.547 W	*Pueraria montana* var. *lobata*	1 October 2009
P. pachyrhizi	TAR 163	Louisiana	Union	32 54.500 N	092 31.257 W	*Pueraria montana* var. *lobata*	1 October 2009
P. pachyrhizi	TAR 164	Louisiana	Caldwell	32 04.851 N	092 01.690 W	*Pueraria montana* var. *lobata*	30 September 2009
P. pachyrhizi	TAR 167	Louisiana	West Baton Rouge	30 46.000 N	091 29.171 W	*Pueraria montana* var. *lobata*	8 October 2009
P. pachyrhizi	TAR 168	Louisiana	West Feliciana	30 55.882 N	090 58.129 W	*Pueraria montana* var. *lobata*	7 October 2009
P. pachyrhizi	TAR 170	Louisiana	St. Helena	30 55.829 N	090 39.603 W	*Pueraria montana* var. *lobata*	7 October 2009
P. pachyrhizi	TAR 172	Alabama	NA	NA	NA	*G. max*	10 October 2009
P. pachyrhizi	TAR 188	Georgia	Berrien	NA	NA	*G. max*	NA
P. pachyrhizi	TAR 190	Tennessee	Madison	NA	NA	*G. max*	12 October 2009
P. pachyrhizi	TAR 224	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	2005
P. pachyrhizi	PP-1	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-2	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-4	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-5	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-6	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-9	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-11	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-12	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-13	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-15	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. pachyrhizi	PP-16	Louisiana	East Baton Rouge	30 36.0168 N	091 17.104 W	*G. max*	12 September 2017
P. tecta	MCA 2965/ BPI 871066	Hawaii	Big Island	19 26.281 N	155 18.334 W	*Commelina* sp.	5 August 2005
P. tecta	JRH 476/ BPI 863978	Puerto Rico	Adjuntas	18 8.788 N	66 43.331 W	*Commelina* sp.	7 December 2004

[a] Locations and dates collected for isolates of multiple species of *Phakopsora* reported in this study. NA = not available. BPI = Specimen collection number deposited in the Fungal Databases, U.S. National Fungus Collections (https://nt.ars-grin.gov/fungaldatabases/specimens/specimens.cfm).

and from cluster assignments (K = 18) specified by the color of each tree tip (Kamvar et al. 2014).

Intragenomic ITS copy variation. To determine the degree of ITS polymorphism across multiple copies of *P. pachyrhizi* present in a single individual, DNA was extracted under forensic conditions from 11 individual sori from 11 different soybean leaves that were collected in 2017 from the Ben Hur Research Station in Baton Rouge, Louisiana (Table 2). DNA from each sorus was extracted, amplified with primers ITSPP5/ITS4u (Freire et al. 2008; Pfunder et al. 2001), and reamplified with nested primers ITS1F/ITS PP3 (Freire et al. 2008; Gardes and Bruns 1993). Polymorphic sites were determined from chromatograms analyzed with Sequencher version 4.1.4 (Gene Codes Corp.). An example chromatogram of isolate PP2 is provided to demonstrate the degree of polymorphisms in each 584- to 629-bp product (Supplementary Figs. S4, S5, and S6).

Results

Specimen collection and DNA extraction. DNA was extracted, and concentrations >20 ng were retained for downstream analysis and stored at −80°C.

Evaluation of previously developed *P. pachyrhizi*-specific PCR and qPCR assays. *Tests for false-positive reactions.* The Frederick et al. (2002) assay using the Ppm1/Ppa2 primers with a FAM probe detected 80% of *P. meibomiae* samples and 100% of *P. pachyrhizi* samples (Table 3). The assay did not detect the other five tested species (Table 3).

The Barnes et al. (2009) assay detected other species of *Phakopsora* (Table 3). PCR with external primers (ITS1rustF4a and Ppa2) amplified only samples of *P. pachyrhizi*. However, the nested qPCR amplified false positives for all other species tested. PCR products at different dilutions had various positive results as shown in Table 3.

The percent match of primer and probe sequences to *P. pachyrhizi* and other species of *Phakopsora* (publicly available on GenBank as of August 2018) were compared, revealing a moderately high potential for false-positive detection of SBR using current diagnostic primers (Fig. 3). For example, primers and probes Ppa2, Pme1, Pme2, Ppm1, Ppm2, FAM probe, VIC probe, ITS1rustF10d, ITS1rustR3c, ITS1rustR3d, ITS1rustF4a, and ITSPP3 had an 80% or greater match with several other species of *Phakopsora* (Fig. 3).

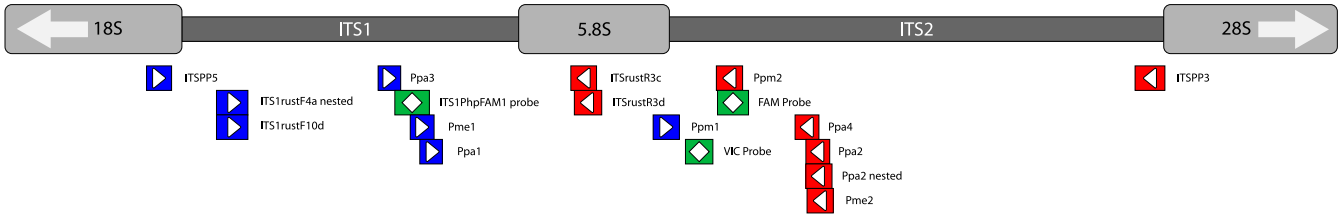
Tests for false-negative reactions. We tested primers used by Barnes et al. (2009) and determined that they amplified all variants of cloned ITS amplicons from *P. pachyrhizi*. Primers ITSPP5, ITS1rustF4a, ITS1rustF10d, Ppa1, Pme1, Ppa4, Ppa2, and Ppme2 and probe ITS1PhpFAM1 were placed in regions containing ≥2 single nucleotide polymorphisms (SNPs) or indels across the diversity of ITS variants of *P. pachyrhizi*. Primers ITSrustR3d, ITSrust3Rc, Ppm1, Ppm2, Ppa3, and ITSPP3 were placed in areas with one SNP or indel across the diversity of ITS variants of *P. pachyrhizi*.

Comparison of ITS variants of *P. pachyrhizi* from a global collection. *Identifying variation of ITS in the United States.* Of the *P. pachyrhizi* isolates collected from the southern United States, 18 isolates were successfully cloned to generate 108 clonal DNA copies. These isolates were shown to have ITS variation among themselves, representing 72 unique copies. Their sequences were compared with a global analysis of *P. pachyrhizi* ITS sequences available on GenBank (Fig. 4), revealing variation among single copies of ITS as a poor predictor of geographic provenance.

Determining whether variants of P. pachyrhizi *are monophyletic.* A maximum likelihood tree constructed from single-copy ITS sequences demonstrates that, despite intraspecific and intragenomic variation, *P. pachyrhizi* is a monophyletic group. *P. pachyrhizi* is inferred as sister to *P. meibomiae* within the confines of our sampling.

Analysis of global ITS copy variation. Our observation that copies of ITS vary within genomes of *P. pachyrhizi* prevents us from drawing conclusions on geographic structure, as done by Freire et al. (2008) and Jorge et al. (2015) (Fig. 4). *K*-means clustering of genotypes across a global data set of ITS sequences shows that genotypes of ITS do not cluster by geography (Fig. 4). However, no conclusions about geographic structure can be drawn from Figure 4 because of high ITS polymorphism both within single genomes and among different fungal individuals and due to unknown genomic positions of each ITS copy (Fig. 1).

Intragenomic ITS copy variation. To confirm that observed intragenomic polymorphism did not stem from mixed infections from several *P. pachyrhizi* spores, DNA was extracted from single sori, which arise from a single, dikaryotic urediniospore. Intragenomic polymorphisms in the ITS region were detected via Sanger sequencing of DNA amplified from single sori. On average, 32% (ranging from 18 to 43% across DNA extracted from 11 individual sori) of loci were polymorphic. There are multiple peaks in the chromatogram found in isolate PP2, as an example of nucleotide diversity found across all *P. pachyrhizi* sori used in this study.



Primer Reference	Primer Type	Primer/Probe Name	Sequence
Frederick et al. 2002		Ppa1	5'-TAAGATCTTTGGGCAATGGT-3'
		Ppa2♦	5'-GCAACACTCAAAATCCAACAAT-3'
		Ppa3	5'-CCCATTTAATTGGCTCATTG-3'
		Ppa4♦	5'-TCAAAATCCAACAATTTCCC-3'
		Pme1	5'-GAAGTTTTTGGGCAAATCAC-3'
		Pme2♦	5'-GCACTCAAAATCCAACATGC-3'
		Ppm1	5'-GCAGAATTCAGTGAATCATCAAG-3'
		Ppm2♦	5'-CTCAAACAGGTGTACCTTTTGG-3'
		FAM probe	5'-CCAAAAGGTACACCTGTTTGAGTGTCA-3'
		VIC probe	5'-TGAACGCACCTTGCACCTTTTGGT-3'
Barnes & Szabo 2007 Barnes et al. 2009	External Primer	ITS1rustF10d	5'-TGAACCTGCAGAAGGATCATTA-3'
		ITS1rustR3c†♦	5'-TGAGAGCCTAGAGATCCATTGTTA-3'
	External Primer	ITSrustR3d♦	5'-TGTGAGAGCCTAGAGATCCATTG-3'
	Nested Primer	ITS1rustF4a	5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3'
	Nested Primer	Ppa2 nested*♦	5'-GCAACACTCAAAATCCAACAAT-3'
		ITS1PhpFAM1 probe	5'-TCATTGATTGATAAGATCTTTGGGCAATGG-3'
Freire et al. 2008		ITSPP5	5'-GCAACGGCACTTTACTGGCTC-3'
		ITSPP3♦	5'-GTTCAGTGGGTAGTCTCACCTGATT-3'

Fig. 2. Top, map of each primer and probe to ITS of *Phakopsora* spp. Right facing triangles label forward primers, left facing triangles label reverse primers, and diamonds label probes. Bottom, sequences for each primer and probe. * indicates a primer that is used as an external or nested primer. ♦ indicates a reverse compliment primer sequence, as listed. † indicates a primer that is not specific to *P. pachyrhizi*.

Discussion

The ITS region varies intraspecifically but also within the genomes of *P. pachyrhizi*. Based on sequences from 72 unique copies of ITS from 18 isolates from the southern United States, along with an additional 139 unique copies obtained from a global pool of 757 sequences from GenBank, it was possible to identify 211 unique copies of the ITS region of *P. pachyrhizi* (of 865 sequences; Fig. 4). In addition, this study provides evidence that sori produced from single urediniospores contain variable copies of the ITS region, with identity among sequences varying, on average, by 32%.

As a result of variation in the ITS region, current data from single copies of ITS prevent inferences on the global migration of *P.*

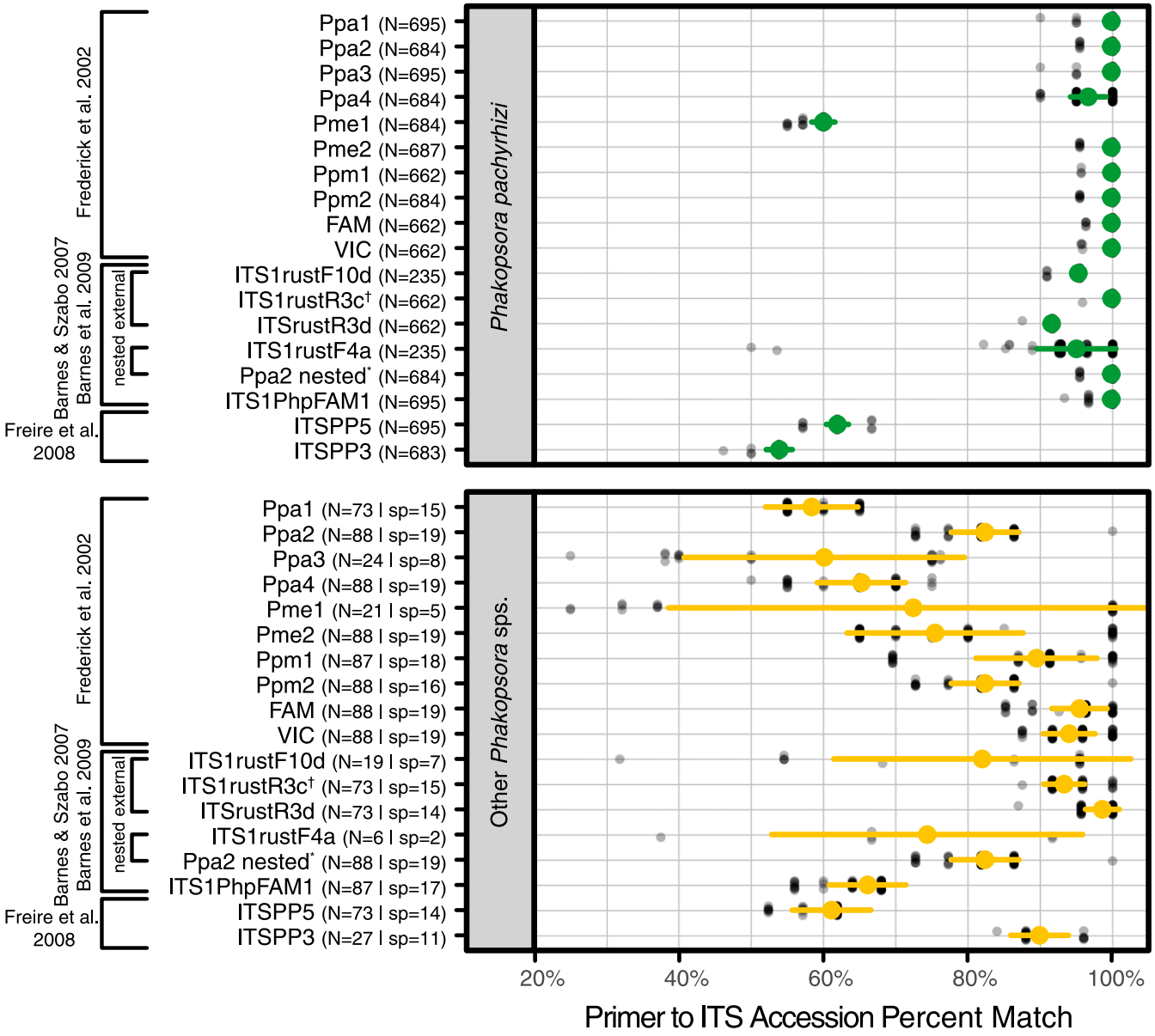


Fig. 3. Nucleotide matches to aligned primers and probes of 867 *Phakopsora pachyrhizi* single copy ITS sequences (top), and 90 single copy ITS sequences of 19 other species of *Phakopsora* (bottom). "N" is the number of sequences tested, "sp" is the number of species tested, "external" indicates primers were used as external primers, and "nested" indicates primers were used as nested primers with the corresponding external primers. * indicates primer was used in different assays. † indicates primers are not specific to *P. pachyrhizi* and not intended for detection of *P. pachyrhizi*.

Table 3. Percentage of positive detection of other species of *Phakopsora* from quantitative polymerase chain reaction analysis

Publication/dilution of DNA	*P. arthuriana*	*P. crotonis*	*P. gossypii*	*P. meibomiae*	*Cerotelium fici* (syn: *P. nishidana*)	*P. pachyrhizi*	*P. tecta*
Number of samples examined	2	1	1	5	1	2	4
Frederick et al. (2002)[a]							
1:1	0	NT	0	80	0	100	0
Barnes et al. (2009)[a]							
1:1	100	100	100	100	100	100	100
1:10	0	100	100	40	100	100	25
1:100	50	100	0	20	0	100	75
1:1,000	0	0	0	20	0	100	0

[a] Percentage of positive detection out of the total samples available, with a cycle threshold of 30 or below. NT = not tested.

pachyrhizi. Comparing a randomly selected single-copy of ITS from many variants in a single genome can lead to flawed conclusions if the genomic position of a given copy is not considered (Fig. 1). Consequently, there is little possibility of delineating the population structure of *P. pachyrhizi* from single-copy ITS, whose genetic dissimilarity is exceedingly difficult to decouple from the arbitrary isolation of ITS copies from the genome with conventional PCR and cloning protocols.

ITS offers some utility in molecular detection of SBR, although caution should be exerted in selecting primers, and contamination from other *Phakopsora* species must be considered. In this study, previously employed diagnostic tools were evaluated for the risk of false-negative and false-positive detection of *P. pachyrhizi*, by both reproducing published protocols and testing primer specificity in silico. Primers specific to *P. pachyrhizi* (presented in Fig. 3) that target binding regions with more than two SNPs are typically considered to be at risk for false-negative detection (Cha and Thilly 1993). However, SBR-specific primers designed by Barnes et al. (2009) and Frederick et al. (2002) that target variable ITS binding regions resulted in successful amplification in all cases.

False-positive identification is a much greater concern when using currently available primers intended to detect *P. pachyrhizi* (Fig. 3). In particular, the primers designed by Barnes et al. (2009) are at risk of false-positive detection for ≥5 species of *Phakopsora* and one species of *Cerotelium*. The Frederick et al. (2002) assay, although less prone to false-positive detection of *P. pachyrhizi*, still shows potential for misidentification, especially if used in aerobiological studies. The efficacy of the Frederick et al. (2002) assay to diagnose leaf tissue infected with *P. pachyrhizi* is corroborated by our results. In addition, *P. meibomiae* was successfully distinguished from SBR, but only with certain primer combinations and with cycle threshold values over 25, in accordance with the original publication (Frederick et al. 2002). There is little need to improve the Frederick et al. (2002) assay, especially when *P. pachyrhizi* and *P. meibomiae* are isolated from leguminous leaf tissue. However, the inability of primers to reliably distinguish SBR from other *Phakopsora* species by both the Barnes et al. (2009) and Frederick et al. (2002) assays highlights the particular risk of using available primer sets on DNA extracted from spores collected in rainwash material, rather than infected leguminous tissue, on which only *P. pachyrhizi* and *P. meibomiae* are observed.

New candidates for diagnostic markers are likely to become available as a greater number of rust fungal genomes become publicly available. However, it is critical that intraspecific and intragenomic variability be considered in the design of new primers and probes for *P. pachyrhizi* and other rust fungi. The main obstacle to the development of new diagnostic markers is a lack of data for other DNA

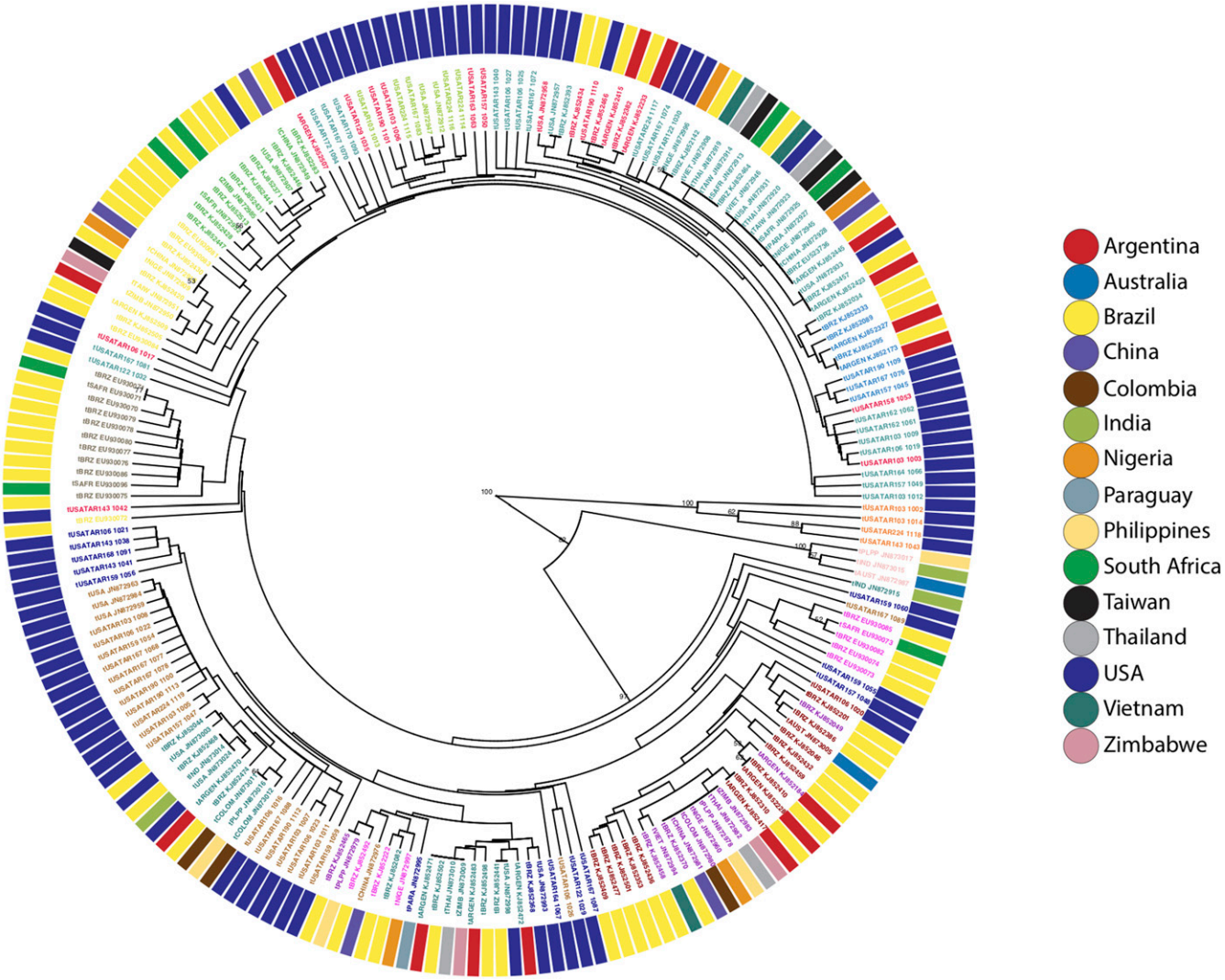


Fig. 4. An unweighted pair group method with arithmetic mean tree of 211 clonal, single-copy internal transcribed spacer (ITS) sequences from global samples of *Phakopsora pachyrhizi*. Tree topology is calculated from an absolute dissimilarity matrix (pairwise Nei's genetic distance), and bootstraps are shown for values >50. Tip labels are shaded according to principal component analysis-based *k*-means hierarchical clustering after removing identical sequences from each population. Outer ring shades denote the country of origin of each ITS copy.

regions (Feau et al. 2011; McTaggart and Aime 2018; Persoons et al. 2014; Uppalapati et al. 2013; Virtudazo et al. 2001). Stockinger et al. (2010) suggested dual or multiple loci for identification of species, such as ITS and 28S (Groenewald et al. 2011; Stockinger et al. 2010), RNA polymerase B2, β-tublin 2, and elongation factor 1 (O'Donnell et al. 2012), or topoisomerase I and phosphoglycerate kinase (Al-Hatmi et al. 2016). Essential to establishing a reliable set of loci to identify *P. pachyrhizi* and other rust species will be their ability to consistently discriminate between species and show minimal intraspecific and intragenomic variation (when multiple copies are present within the genome).

Disease forecasting based on molecular diagnostics has the potential to mitigate crop loss in advance of disease incidence and improve the timing of fungicide application and overall use. However, more knowledge is needed to provide accurate, reliable, and functional primers and probes for rust fungi that avoid the risk of false-positive and false-negative species detection.

Acknowledgments

The authors thank P. A. Bollich and C. Robertson for some of the infected soybean and kudzu samples used in this study; G. Heller, M. Hoy, M. Keller-Pearson, A. Pringle, and M. Toome-Heller for technical advice; B. Kennedy for assistance with DNA extractions; and L. Urbatsch for identification of plant specimens. Finally, the authors thank the two anonymous reviewers and senior editor for their valuable comments, which greatly improved this manuscript.

Literature Cited

Aime, M. C. 2006. Towards resolving family-level relationships in rust fungi (Uredinales). Mycoscience 47:112-122.

Al-Hatmi, A. M., Van Den Ende, A. G., Stielow, J. B., Van Diepeningen, A. D., Seifert, K. A., McCormick, W., Assabgui, R., Gräfenham, T., Sybren De Hoog, G., and Levesque, C. A. 2016. Evaluation of two novel barcodes for species recognition of opportunistic pathogens in *Fusarium*. Fungal Biol. 120:231-245.

Alfieri, S. A., Jr., Landon, K. R., Wehlburg, C., and Kimbrough, J. W. 1984. Index of Plant Diseases in Florida (Revised). Florida Dept. Agric. Consumer Serv. Div. Plant Ind. Bull. 11:1-389.

Allen, T. W., Bradley, C. A., Sisson, A. J., Byamukama, E., Chilvers, M. I., Coker, C. M., Collins, A. A., Damicone, J. P., Dorrance, A. E., Dufault, N. S., Esker, P. D., Faske, T. R., Giesler, L. J., Grybauskas, A. P., Hershman, D. E., Hollier, C. A., Isakeit, T., Jardine, D. J., Kemerait, R. C., Kleczewski, N. M., Koenning, S. R., Kurle, J. E., Malvick, D. K., Markell, S. G., Mehl, H. L., Mueller, D. S., Mueller, J. D., Mulrooney, R. P., Nelson, B. D., Newman, M. A., Osborne, L., Overstreet, C., Padgett, G. B., Phipps, P. M., Price, P. P., Sikora, E. J., Smith, D. L., Spurlock, T. N., Tande, C. A., Tenuta, A. U., Wise, K. A., Wrather, J. A., and Young-Kelly, H. 2017. Soybean yield loss estimates due to diseases in the United States and Ontario, Canada, from 2010 to 2014. Plant Health Prog. 18:19-27.

Anonymous. 1960. Index of plant diseases in the United States. U.S.D.A. Agric. Handb. 165:1-153.

Badotti, F., de Oliveria, F. S., Garcia, C. F., Vaz, A. B. M., Fonseca, P. L. C., Nahum, L. A., Oliveira, G., and Góes-Neto, A. 2017. Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). BMC Microbiol. 17:42.

Barnes, C. W., Szabo, L. J., and Bowersox, V. C. 2009. Identifying and quantifying *Phakopsora pachyrhizi* spores in rain. Phytopathology 99:328-338.

Beenken, L. 2014. Pucciniales on *Annona* (Annoanceae) with special focus on the genus *Phakopsora*. Mycol. Prog. 13:791-809.

Bonde, M. R., Nester, S. E., Austin, C. N., Stone, C. L., Frederick, R. D., Hartman, G. L., and Miles, M. R. 2006. Evaluation of virulence of *Phakopsora pachyrhizi* and *P. meibomiae* isolates. Plant Dis. 90:708-716.

Buritica, P. 1999. La familia Phakopsoraceae en el neotropico III, generos: *Batistopsora* y *Phakopsora*. Rev. Acad. Colomb. Cienc. 23:271-305.

Cha, R. S., and Thilly, W. G. 1993. Specificity, efficiency, and fidelity of PCR. PCR Methods Appl. 3:S19-S29.

Cooke, W. B. 1971. The 1967 foray in Texas. Mycologia 63:1063-1067.

Cummins, G. B. 1962. Supplement to Arthur's Manual of Rusts in United States and Canada. Vol. 24. Hafner Publishing Company, New York, NY.

Cummins, G. B. 1964. Uredinales of the Big Bend National Park and adjacent areas of Texas. Southwest. Nat. 8:181-195.

Cummins, G. B. 1979. Annotated, illustrated, host index of Sonoran Desert rust fungi. Mycotaxon 10:1-20.

Cummins, G. B., and Stevenson, J. A. 1956. A check list of North American rust fungi (Uredinales). Plant Dis. Rep. (Suppl 240):109-192.

de Jensen, C. E., Harmon, C. L., and Vitoreli, A. 2013. First report of Asian soybean rust caused by *Phakopsora pachyrhizi* in Puerto Rico. Plant Dis. 97:1378.

Eickbush, T. H., and Eickbush, D. G. 2007. Finely orchestrated movements: Evolution of the ribosomal RNA genes. Genetics 175:477-485.

Esker, P. D., Sparks, A. H., Bates, M., Dall'Acqua, W., Frank, E. E., Huebel, L., Segovia, V., and Garrett, K. A. 2007. Modeling dispersal gradients. Plant Health Instr.

Farr, D. F., and Rossman, A. Y. 2018. U.S. Department of Agriculture Agricultural Research Service Fungal Database, U.S. National Fungus Collections. http://nt.ars-grin.gov/fungaldatabases/

Feau, N., Vialle, A., Allaire, M., Maier, W., and Hamelin, R. C. 2011. DNA barcoding in the rust genus *Chrysomyxa* and its implications for the phylogeny of the genus. Mycologia 103:1250-1266.

Fletcher, J., Luster, D., Bostock, R., Burans, J., Cardwell, K., Gottwald, T., McDaniel, L., Royer, M., and Smith, K. 2010. Emerging infectious plant diseases. Pages 337-366 in Emerging Infections 9. W. Scheld, M. Grayson, and J. Hughes, eds. American Society of Microbiology Press, Washington, DC.

Frederick, R. D., Snyder, C. L., Peterson, G. L., and Bonde, M. R. 2002. Polymerase chain reactions assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae*. Phytopathology 92:217-227.

Freire, M. C. M., da Silva, M. R., Zhang, X., Almeida, Á. M. R., Stacey, G., and de Oliveira, L. O. 2012. Nucleotide polymorphism in the 5.8S nrDNA gene and internal transcribed spacers in *Phakopsora pachyrhizi* viewed from structural models. Fungal Genet. Biol. 49:95-100.

Freire, M. C. M., de Oliveira, L. O., Almeida, Á. M. R., Schuster, I., Moreira, M. A., Liebenberg, M. M., and Mienie, C. M. S. 2008. Evolutionary history of *Phakopsora pachyrhizi* (the Asian soybean rust) in Brazil based on nucleotide sequences of the internal transcribed spacer region of the nuclear ribosomal DNA. Genet. Mol. Biol. 31:920-931.

Gallegos, M. L., and Cummins, G. B. 1981. Uredinales (Royas) de Mexico. Pages 1-462 in: Morfologia y taxonomia de Uredinales (Royas) en Mexico. Vol. 2. M. L. Gallegos, ed. Instituto Nacional de Investigaciones Agrícolas, Culiacan, Sinaloa, Mexico.

Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for Basidiomycetes application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Gatto, L., Catanzaro, D., and Milinkovitch, M. C. 2006. Assessing the applicability of the GTR nucleotide substitution model through simulations. Evol. Bioinform. Online 2:145-155.

Gilbertson, R. L., and McHenry, J. 1969. Check list and host index for Arizona rust fungi. Univ. Ariz. Agric. Exp. Sta. Techn. Bull. 186:1-40.

Golan, J. J., and Pringle, A. 2017. Long-distance dispersal of fungi. Microbiol. Spectr. 5:1-24.

Goss, E. M., Tabima, J. F., Cooke, D. E. L., Restrepo, S., Fry, W. E., Forbes, G. A., Fieland, V. J., Cardenas, M., and Grünwald, N. J. 2014. The Irish potato famine pathogen *Phytophthora infestans* originated in central Mexico rather than the Andes. Proc. Natl. Acad. Sci. USA 111:8791-8796.

Gouy, M., Milleret, F., Mugnier, C., Jacobzone, M., and Gautier, C. 1984. ACNUC: A nucleic acid sequence data base and analysis system. Nucleic Acids Res. 12:121-127.

Groenewald, M., Robert, V., and Smith, M. T. 2011. The value of the D1/D2 and internal transcribed spacers (ITS) domains for the identification of yeast species belonging to the genus *Yamadazyma*. Persoonia Mol. Phylogeny Evol. Fungi. 26:40-46.

Grünwald, N. J., Goodwin, S. B., Milgroom, M. G., and Fry, W. E. 2003. Analysis of genotypic diversity data for populations of microorganisms. Phytopathology 93:738-746.

Grünwald, N. J., and Hoheisel, G. A. 2006. Hierarchical analysis of diversity, selfing, and genetic differentiation in populations of the oomycete *Aphanomyces euteiches*. Phytopathology 96:1134-1141.

Hennen, J. F., Figueiredo, M. B., de Carvalho, A. A., Jr., and Hennen, P. G. 2005. Catalogue of the species of plant rust fungi (Uredinales) of Brazil. Jardim Bot. Rio de Janeiro.

Integrated Pest Management–Pest Information Platform for Extension and Education. 2018. Integrated Pest Management–Pest Information Platform for Extension and Education (IPM-PIPE) for Soybean Rust. http://sbr.ipmpipe.org/cgi-bin/sbr/public.cgi

Isard, S. A., Barnes, C. W., Hambleton, S., Ariatti, A., Russo, J. M., Tenuta, A., Gay, D. A., and Szabo, L. J. (2011) Predicting soybean rust incursions into the North American continental interior using crop monitoring, spore trapping, and aerobiological modeling. Plant Dis. 95:1346-1357.

Isard, S. A., Gage, S. H., Comtois, P., and Russo, J. M. 2005. Principles of the atmospheric pathway for invasive species applied to soybean rust. Bioscience 55:851-861.

Isard, S. A., Russo, J. M., and Ariatti, A. 2007. The integrated aerobiology modeling system applied to the spread of soybean rust into the Ohio River valley during September 2006. Aerobiologia 23:271-282.

Jombart, T., and Ahmed, I. 2011. *adegenet 1.3-1*: New tools for the analysis of genome-wide SNP data. Bioinformatics 27:3070-3071.

Jorge, V. R., Silva, M. R., Guillin, E. A., Freire, M. C. M., Schuster, I., Almeida, Á. M. R., and de Oliveira, L. O. 2015. The origin and genetic diversity of the causal agent of Asian soybean rust, *Phakopsora pachyrhizi*, in South America. Plant Pathol. 64:729-737.

Kamvar, Z. N., Tabima, J. F., and Grünwald, N. J. 2014. *Poppr*: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281.

Katoh, K., Rozewicki, J., and Yamada, K. D. 2017. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. doi:10.1093/bib/bbx108

Kelly, H. Y., Dufault, N. S., Walker, D. R., Isard, S. A., Schneider, R. W., Giesler, L. J., Wright, D. L., Marois, J. J., and Hartman, G. L. 2015. From select agent to an established pathogen: The response to *Phakopsora pachyrhizi* (soybean rust) in North America. Phytopathology 105:905-916.

Kiss, L. 2012. Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for Fungi. Proc. Natl. Acad. Sci. 109:E1811.

Kuraku, S., Zmasek, C. M., Nishimura, O., and Katoh, K. 2013. aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Res. 41:W22-W28.

Maddison, W. P., and Maddison, D. R. 2008. Mesquite: A modular system for evolutionary analysis. Evolution 62:1103-1118.

McCain, J. W., Hennen, J. F., and Ono, Y. 1990. New host species and state distribution records for North American rust fungi (Uredinales). Mycotaxon 39:281-300.

McTaggart, A. R., and Aime, M. C. 2018. The species of *Coleosporium* (Pucciniales) on *Solidago* in North America. Fungal Biol. 122:800-809.

Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457-477.

Nei, M., and Rooney, A. P. 2005. Concerted and birth-and-death evolution of multigene families. Annu. Rev. Genet. 39:121-152.

Nelson, M. R., Orum, T. V., Jaime-Garcia, R., and Nadeem, A. 1999. Applications of geographic information systems and geostatistics in plant disease epidemiology and management. Plant Dis. 83:308-319.

Nilsson, R. H., Kristiansson, E., Ryberg, M., and Hallenberg, N. 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol. Bioinforma. 4:193-201.

O'Donnell, K., Humber, R. A., Geider, D. M., Kang, S., Park, B., Robert, V. A., Crous, P. W., Johnston, P. R., Aoki, T., Rooney, A. P., and Rehner, S. A. 2012. Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and Fusarium MLST. Mycologia 104:427-445.

Okane, I., and Ono, Y. 2018. Phylogenetic study of indigenous grapevine leaf rust fungi in North America and biological identity of an invasive grapevine leaf rust fungus in Brazil. Mycoscience 59:99-104.

Ono, Y. 2016. *Phakopsora hornotina*, an additional autoecious rust species on *Melisoma* in the Philippines and the Ryuku, Islands, Japan. Mycoscience 57:71-78.

Ono, Y., Buriticá, P., and Hennen, J. F. 1992. Delimination of *Phakopsora*, *Physopella* and *Cerotelium* and their species on Leguminosae. Mycol. Res. 96:825-850.

Overholts, L. O. 1938. Notes on fungi from the lower Mississippi Valley. Bull. Torrey Bot. Club 65:167-180.

Persoons, A., Morin, E., Delaruelle, C., Payen, T., Halkett, F., Frey, P., De Mita, S., and Duplessis, S. 2014. Patterns of genomic variation in the poplar rust fungus *Melampsora larici-populina* identify pathogenesis-related factors. Front. Plant Sci. 5:1-16.

Pfunder, M., Schürch, S., and Roy, B. 2001. Sequence variation and geographic distribution of pseudoflower-forming rust fungi (*Uromyces pisi* s. lat.) on *Euphorbia cyparissias*. Mycol. Res. 105:57-66.

Plantegenest, M., Le May, C., and Fabre, F. 2007. Landscape epidemiology of plant diseases. J. R. Soc. Interface 4:963-972.

Pota, S., Chatasiri, S., Unartngam, J., Yamaoka, Y., Hosaka, K., and Ono, Y. 2015. Taxonomic identity of a *Phakopsora* fungus causing the grapevine leaf rust disease in Southeast Asia and Australasia. Mycoscience 56: 198-204.

Schoch, C. L., and Seifert, K. A. 2012. Reply to Kiss: Internal transcribed spacer (ITS) remains the best candidate as a universal DNA barcode marker for fungi despite imperfections. Proc. Natl. Acad. Sci. 109:E1812.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., and Chen, W.; Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc. Natl. Acad. Sci. U. S. A. 109:6241-6246.

Schumann, G. L., and Leonard, K. J. 2000. Stem rust of wheat (black rust). Plant Health Instr.

Sikora, E. J., Allen, T. W., Wise, K. A., Baniecki, J., Bergstrom, G., Bradley, C., Bond, J., Brown-Rytlewski, D., Chilvers, M., Coker, C., Damicone, J., DeWolf, E., Dorrance, A., Dufault, N., Esker, P., Faske, T., Giesler, L., Grau, C., Golod, J., Grybauskas, A., Franc, G., Hammerschmidt, R., Hartman, G. L., Henn, A., Hershman, D., Hollier, C., Isakeit, T., Isard, S., Jacobson, B., Jardine, D., Kemerait, B., Koenning, S., Malvick, D., Markell, S., Marois, J. J., Monfort, S., Mueller, D., Mueller, J., Mulrooney, B., Newman, M., Osborne, L., Padgett, G. B., Ruden, B. E., Rupe, J., Schneider, R., Schwartz, H., Shaner, G., Singh, S., Stromberg, E., Sweets, L., Tenuta, A., Trippett, C., Vaiciunas, S., Yang, X.B., Young- Kelly, H., and Zidek, J. 2014. A coordinated effort to manage soybean rust in North America: A success story in soybean disease monitoring. Plant Dis. 98:864-875.

Silva, D. C., Yamanaka, N., Brogin, R. L., Arias, C. A., Nepomuceno, A. L., Di Mauro, A. O., Pereira, S. S., Nogueira, L. M., Passianotto, A. L., and Abdelnoor, R. V. 2008. Molecular mapping of two loci that confer resistance to Asian rust in soybean. Theor. Appl. Genet. 117:57-63.

Simon, U. K., and Weiß, M. 2008. Intragenomic variation of fungal ribosomal genes is higher than previously thought. Mol. Biol. Evol. 25:2251-2254.

Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312-1313.

Stockinger, H., Krüger, M., and Schüßler, A. 2010. DNA barcoding of arbuscular mycorrhizal fungi. New Phytol. 187:461-474.

Uppalapati, S. R., Serba, D. D., Ishiga, Y., Szabo, L. J., Mittal, S., Bhandari, H. S., Bouton, J. H., Mysore, K. S., and Saha, M. C. 2013. Characterization of the rust fungus, *Puccinia emaculata*, and evaluation of genetic variability for rust resistance in switchgrass populations. BioEnergy Res. 6:458-468.

Virtudazo, E. V., Nakamura, H., and Kakishima, M. 2001. Ribosomal DNA-ITS sequence polymorphism in the sugarcane rust, *Puccinia kuehnii*. Mycoscience 42:447-453.

Williams, R. H., Ward, E., and McCartney, H. A. 2001. Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Appl. Environ. Microbiol. 67:2453-2459.

Yohem, K. H., Cummins, G. B., and Gilbertson, R. L. 1985. Revised list and host index of Arizona rust fungi. Mycotaxon 22:451-468.

Yorinori, J. T., Paiva, W. M., Frederick, R. D., Costamilan, L. M., Bertagnolli, P. F., Hartman, G. E., Godoy, C. V., and Nunes, J., Jr. 2005. Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. Plant Dis. 89:675-677.

Young, H. M., Marois, J. J., Wright, D. L., Narváez, D. F., and O'Brien, G. K. 2011. Epidemiology of soybean rust in soybean sentinel plots in Florida. Plant Dis. 95:744-750.

Zhang, X. C., Freire, M. C. M., Le, M. H., de Oliveira, L. O., Pitkin, J. W., Segers, G., Concibido, V. C., Baley, G. J., Hartman, G. L., Upchurch, G., Pedley, K. F., and Stacey, G. 2012. Genetic diversity and origins of *Phakopsora pachyrhizi* isolates in the United States. Asian J. Plant Pathol. 6:52-65.