

Phylogenetic analyses of eight isolates of *Phakopsora meibomia* collected in Puerto Rico^{1,2}

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ABSTRACT

American soybean rust (AmSR) caused by *Phakopsora meibomia* (Arthur) Arthur is limited to certain areas of the Western Hemisphere. It is not known to cause severe losses to economically important crops. *Phakopsora meibomia* naturally infects 42 species of legumes of the subfamily Papilionoidea. During 2008, eight isolates of AmSR infecting *Lablab purpureus* were collected in the central mountainous area of Puerto Rico. Molecular phylogenetic analyses of the internal transcribed spacer (ITS) region, including 5.8S ribosomal DNA (rDNA), were conducted. The topology of the trees generated by parsimony and by Neighbor-Joining methods was similar. Isolates were identified as *Phakopsora meibomia*, with 98 and 100% identity. These isolates formed a monophyletic group. Asian Soybean Rust, *Phakopsora pachyrhizi*, the most closely related species, formed a second clade. Other rust species of the genera *Uromyces*, *Puccinia*, *Tranzschelia*, *Hemileia*, *Ustilago* smut, and the edible mushroom *Boletus* were included in the analyses as outgroups. *Phakopsora* species grouped in the Pucciniaceae family formed a sister clade to Phakopsoraceae and Uropyxidaceae families.

Key words: American soybean rust, phylogenetic analyses

RESUMEN

Análisis filogenético de la roya americana de la soya, *Phakopsora meibomia*

La roya americana de la soya (AmSR), causada por *Phakopsora meibomia* (Arthur) Arthur, se encuentra limitada a pocas áreas del Hemisferio Occidental. No se conocen pérdidas severas causadas por este patógeno en cultivos de importancia económica. *Phakopsora meibomia* infecta naturalmente 42 especies de leguminosas de la subfamilia Papilionoidea. Durante el 2008, se colectaron ocho aislados de AmSR

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infectando *Lablab purpureus* en la cordillera central de Puerto Rico. Se realizó análisis molecular filogenético de la región interna transcrita (ITS), incluyendo la región 5.8S ribosomal (rADN). La topología del árbol generado por los métodos de parsimonia y Neighbor-Joining fue similar. Los aislados se identificaron como *Phakopsora meibomiae* con un porcentaje de identidad entre el 98 y 100%. Estos aislados fueron agrupados en un grupo monofilético. *Phakopsora pachyrhizi* fue la especie más relacionada, y formó un segundo clado. Otras especies de royas de los géneros *Uromyces*, *Puccinia*, *Tranzschelia*, *Hemileia*, el carbón *Ustilago* y la seta comestible *Boletus* se incluyeron en el análisis como grupos distantes. Las especies de *Phakopsora* agrupadas en la familia Pucciniaceae forman un clado hermano con las familias Phakopsoraceae y Uropyxidaceae.

Palabras clave: Ruya americana de la soya, análisis filogenético

INTRODUCTION

Soybean rust is caused by two *Phakopsora* species, *P. meibomiae* (Arthur) Arthur named American Soybean Rust (AmSR), and *P. pachyrhizi* Sydow commonly named Asian Soybean Rust (ASR) (Frederick et al., 2002; Posada-Buitrago and Frederick, 2005; Pivonia and Yang, 2004; Villavicencio et al., 2007; Yorinori et al., 2005). *Phakopsora meibomiae* is endemic to Central America, South America and the Caribbean (Bonde et al., 2006; Ono et al., 1992) and was reported in wild legumes under the name of *Uredo vignae* Bresadola (Ono et al., 1992). Earlier studies of rust infection in legumes in the Caribbean region reported *Uredo concors* Arthur and *Physopella concors* Arthur as the causal agents of the disease (Arthur, 1915; Arthur, 1917). In the Americas, AmSR was described in 1917 in Mexico, in 1940 in Guatemala, and later in 1976 in Costa Rica infecting wild legumes (Cummins, 1943; Bromfield, 1984). *Phakopsora meibomiae* has been reported in 15 wild legume species of the subfamily Papilionoidea (Ono et al., 1992).

Phakopsora pachyrhizi was first recorded in Japan in 1903 (Bromfield, 1984). In the Americas it was reported for the first time in Paraguay in 2001 (Yorinori et al., 2005) and was rapidly disseminated to Argentina, Bolivia, Brazil and Colombia (Rossi, 2003; Hartman et al., 2004; Pan et al., 2006). Asian soybean rust was reported for the first time in 2004 in the continental United States in Louisiana (Schneider et al., 2005).

In 1976 an outbreak of soybean rust was observed in Adjuntas, Puerto Rico, infecting scarlet runner bean and soybean (Vakili and Bromfield, 1976). From 1976 to 1979, field surveys of endemic legumes of Puerto Rico confirmed the presence of rust infecting *Lablab purpureus*, *Vigna luteola*, *Teramnus uncinatus*, *Phaseolus lunatus*, *P. vulgaris*, *Crotalaria pallida* and *Macroptilium lathyroides* (Vakili, 1979; Vakili,

1981). The pathogen was erroneously identified as *P. pachyrhizi* until Ono et al. (1992) reclassified it as *P. meibomiae* on the basis of the telial stage found in samples collected in the Americas, including samples collected by Vakili in Puerto Rico.

Both *Phakopsora* species infect a broad range of plant species. Even though *Phakopsora* species produce only the anamorph stage, they could produce telia under favorable conditions (Ono et al., 1992). *Phakopsora meibomiae* naturally infects 42 species of legumes in 19 genera. Under controlled conditions, 18 additional species in 12 genera are reported to be infected by this pathogen. *Phakopsora pachyrhizi* naturally infects 31 species of legumes in 17 genera; under controlled conditions 60 species in 26 additional genera have been infected. For both species, 24 plant species in 19 genera have been reported to be hosts (Frederick et al., 2002; Ono et al., 1992). Differences in virulence between *P. meibomiae* and *P. pachyrhizi* have been documented (Bonde et al., 2006).

Morphological differences between teleomorphs of the two *Phakopsora* species are similar with groupings based on the sequence of the internal transcribed spacer (ITS) (Frederick et al., 2002). Based on nucleotide sequences, specific primers were developed for their rapid and accurate identification using both conventional and real-time PCR (Frederick et al., 2002; Lamour et al., 2006).

Phylogenetic analyses based on DNA regions have served to define relationships among different organisms (Chatasiri and Ono, 2008). The use of the ITS region of the rDNA of the *Puccinia monoica* complex (Roy et al., 1998), *Uromyces pisi* complex (Pfunder et al., 2001), *Melampsora epitea* (Smith et al., 2004), *Puccinia hemerocallidis* (Chatasiri et al., 2006), *Puccinia andropogonis* and *Puccinia coronata* (Szabo, 2006), among others, has been successful in phylogenetic analyses of rust taxa.

The objective of this study was to identify and characterize eight isolates of AmSR collected in the central mountainous region of Puerto Rico, on the basis of the sequence of the ITS region of their rDNA.

MATERIALS AND METHODS

Selected isolates. Eight isolates of *P. meibomiae* infecting *Lablab purpureus* collected during 2008 were used in the present study (Table 1). All specimens were deposited in the Herbarium of the Plant Disease Clinic at the Juana Díaz Substation, University of Puerto Rico.

For ITS analyses, GenBank sequences of *P. pachyrhizi* (AF333488), *Uromyces vignae* (AB115736), *U. appendiculatus* (DQ411531), *Puccinia hordei* (DQ460723), *P. coronata* f. sp. *avenae* (EU014044), *Hemileia*

TABLE 1.—*Isolates of Phakopsora meibomiaae used in the present study.*

Isolate No.	Site of Collection	Location	GenBank accession No.
LP17	Jayuya	N 18°11.576'; W 066°30.965'	GQ146460
LP18	Adjuntas	N 18°11.162'; W 066°45.411'	GQ146461
LP19	Barranquitas	N 18°11.833'; W 066°22.115'	GQ146462
LP20	Aibonito	N 18°08.205'; W 066°17.522'	GQ146463
LP21	Aibonito	N 18°07.492'; W 066°17.480'	GQ146464
LP22	Yahuecas	N 18°11.897'; W 066°47.895'	GQ146465
LP23	Adjuntas	N 18°10.474'; W 066°47.962'	GQ146466
LP24	Adjuntas	N 18°10.855'; W 066°46.123'	GQ146467

vastatrix (EF394132), *Tranzschelia discolor* (EU014071), *Ustilago hordei* (AY345003), and *Boletus mirabilis* (AF335451) were used as the outgroups (Table 2). Additionally, the sequence of *P. meibomiaae* (AF333502) was used as reference.

DNA extraction, polymerase chain reaction and DNA sequencing. Total DNA was extracted from 1 to 5 mg of urediniospores. Urediniospores were deposited in a 2-ml Eppendorf tube with three glass beads of 5-mm diameter (Fisher Scientific, Pittsburgh, PA)⁵. The extraction of DNA was conducted with the DNeasy Plant Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, increasing the incubation time from 15 to 60 minutes. The ITS region, including 5.8S rDNA, was amplified with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Integrated DNA Technologies, Coralville, IA) in a total volume of 20 µl. The PCR reaction contained REExtract-N-Amp PCR ready mix (Sigma-Aldrich, St. Louis, MO) with each primer at a concentration of 20 pmol, and the use of 5 to 50 ng of extracted template DNA. Amplification of extracted DNA was performed in a thermocycler model T 3000 (Biometra, Goettingen, Germany) under the following conditions: 94° C for 1 min initial denaturation, followed by 35 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 1 min, and a final extension for 6 min at 72° C (White et al., 1990; Fernández, 2005). The amplification products were visualized by using a UV transilluminator after electrophoresis in a 1.0% agarose gel stained with ethidium bromide. The PCR products were purified and sequenced at the Sequencing and Genotyping facility of the University of Puerto Rico in Río Piedras (<http://mcc.com.pr>).

⁵Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

TABLE 2.—Previously characterized fungal species and GenBank sequences used in the phylogenetic analyses.

Fungus	Source	GenBank accession No.
<i>Phakopsora meibomiaae</i> isolate Puerto Rico	Frederick, R. D., Snyder, C. L., Peterson, G. L. and Bonde, M. R.	AF333502
<i>Phakopsora pachyrhizi</i> isolate Australia 72-1	Frederick, R. D., Snyder, C. L., Peterson, G. L. and Bonde, M. R.	AF333488
<i>Uromyces vignae</i>	Chung, W. H., Tsukiboshi, T., Ono, Y. and Kakishima, M.	AB115736
<i>Uromyces appendiculatus</i> isolate AFTOL-ID 976 clone 2	Matheny, P. B., Aime, M. C. and Hibbett, D. S.	DQ411531
<i>Puccinia hordei-secalini</i> isolate RN-3	Jafary, H., Szabo, L. J. and Niks, R. E.	DQ460723
<i>Puccinia coronata</i> f. sp. <i>avenae</i> isolate R201 clone 01	Alaei, H., De Backer, M., Nuytinck, J., Maes, M., Hofte, M. and Heungens, K.	EU014044
<i>Hemileia vastatrix</i> isolate CT-9-8	Cristancho, M. A., Escobar, C., Varzea, V. and Ribeiro, A. I.	EF394132
<i>Tranzschelia discolor</i> isolate R218 clone 01	Alaei, H., De Backer, M., Nuytinck, J., Maes, M., Hofte, M. and Heungens, K.	EU014071
<i>Ustilago hordei</i>	Stoll, M., Piepenbring, M., Begerow, D. and Oberwinkler, F.	AY345003
<i>Boletus mirabilis</i>	Berbee, M. L., Inderbitzin, P. and Zhang, G.	AF335451

Alignment and phylogenetic analyses. DNA sequences were aligned and manually edited by using the Sequencher program, version 4.5 (Gibbs and Cockerill, 1995). Sequence data were deposited in GenBank (Table 1). Multiple alignment was performed by using the BioEdit program, version 7.0.4.1 (Hall, 1999). Phylogenetic analyses were performed by using heuristic maximum parsimony and maximum likelihood methods in MEGA, version 4.1 (Tamura et al., 2007). Node support in parsimony analyses was assessed by bootstrapping with 1,000 pseudoreplicates, each with 10 random additions of the included taxa to increase the likelihood of finding the most parsimonious tree. Evolutionary distances for the aligned sequences were computed by using the maximum composite likelihood method and were analyzed by the Neighbor-Joining method. The relative nodal support was calculated by using bootstrapping with 1,000 pseudoreplicates. All positions containing gaps and missing data were eliminated from the analysis.

RESULTS AND DISCUSSION

The partial sequence of both the 18S rRNA gene and the ITS2 region, as well as the complete sequence of the 5.8S rRNA gene and the ITS1 region of the eight isolates, corresponded to *P. meibomiae* with an identity ranging from 98 to 100%. The length of the ITS1 ranged from 214 to 218 bp, whereas the length of the 5.8S ranged from 152 to 154 bp. The coverage of the sequenced region varied from 81 to 100%. The gaps found varied from one to seven base pairs (bp). The length of the sequenced region (partial sequence of both 18S and ITS2; and complete sequence of both 5.8S and ITS1) ranged from 466 to 552 bp (Table 3). Both the ITS region and the intergenic spacer of the nuclear rRNA genes have been used as powerful tools to characterize phyloge-

TABLE 3.—*Identification and molecular characterization of isolates used in the present study.*

Isolate No.	BLAST identification	% Identity ¹	% Coverage ¹	Gaps ¹	Length ²
LP17	<i>Phakopsora meibomiae</i>	99	87	2/463	526
LP18	<i>P. meibomiae</i>	100	99	4/463	492
LP19	<i>P. meibomiae</i>	99	83	1/462	552
LP20	<i>P. meibomiae</i>	99	82	3/435	525
LP21	<i>P. meibomiae</i>	99	86	4/405	466
LP22	<i>P. meibomiae</i>	99	100	3/443	543
LP23	<i>P. meibomiae</i>	98	86	7/430	491
LP24	<i>P. meibomiae</i>	98	81	6/447	543

¹Based on BLAST analyses.

²Length (bp) of 18S rDNA partial sequence, ITS1 and 5.8S rDNA complete sequence and ITS2 partial sequence.

netically different taxonomic groups (White et al., 1990). Furthermore, ribosomal genes present a high number of copies within a single genome, possess variable and conserved regions, and can be amplified and sequenced with universal primers (Henson and French, 1993). Therefore, the ITS region has been used in phylogeny and taxonomy of closely related species such as the Asian grapevine leaf rust, caused by *Phakopsora euvitis* (Chatasiri and Ono, 2008).

Problems associated with DNA extraction and ITS sequencing produced the variation in the number of bp sequenced among the isolates, and these problems have frequently been found during sequencing of the ITS region of biotrophs, such as soybean rust. Chatasari and Ono (2008) found differences in 20 base positions in the ITS2 sequence of *P. euvitis* between the Japanese and the Australian-East Timor isolates. Nevertheless, one Japanese isolate, compared with 18 isolates of the same country, showed one bp difference in the ITS2 region at positions 170 and 367 (Chatasiri and Ono, 2008).

Of the 1,437 aligned characters, 442 were conserved, 946 were variable, and 355 were parsimony informative. The maximum parsimony method generated 113 trees with a tree length of 564 steps, a retention index of 0.701, and a composite index of 0.4885 (Figure 1). The

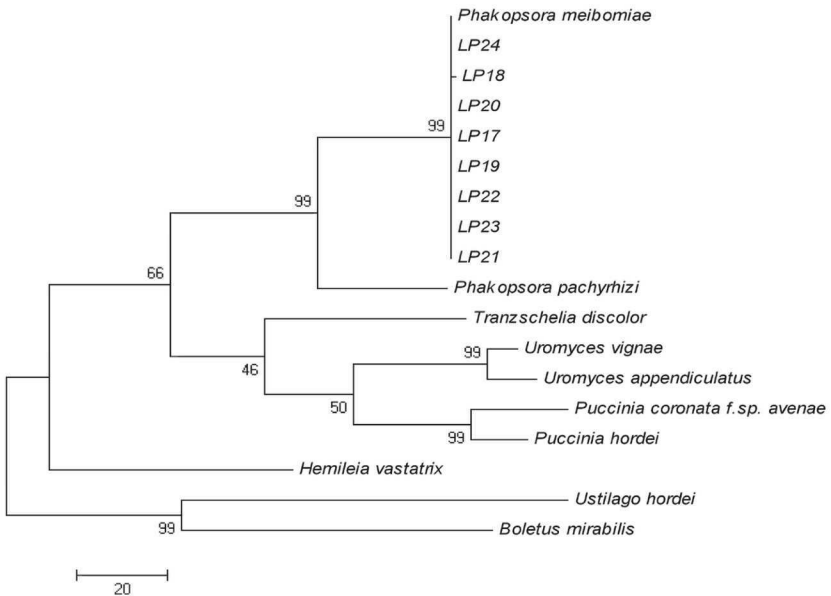


FIGURE 1. Consensus parsimony tree constructed from sequences of the ITS1-5.8S-ITS2 region, fungal species names and isolate numbers are shown in the figure; the bootstrap values calculated from 1,000 replicates are given above and below the branches.

Neighbor-Joining (NJ) tree obtained by the distance method showed a topology identical to the consensus parsimony tree. The sum of the optimal tree branch length was 2.4569 (Figure 2).

Together, eight *P. meibomiae* Puerto Rico isolates formed a sister clade with a bootstrap value of 100. *Phakopsora pachyrhizi* was the most closely related species, forming a second clade. Both species belong to the Phakopsoraceae family. According to Frederick et al. (2002), the comparison of the ITS1 regions of *P. pachyrhizi* and *P. meibomiae* showed 49 nucleotide differences, equivalent to 77.5% identity between the two species. The comparison of the ITS2 regions showed 64 nucleotide differences, representing 68.5% identity between the two *Phakopsora* species. Thus, this molecular differentiation supports the biological and taxonomic distinctness of the two *Phakopsora* species (Ono et al., 1992).

The species grouped in the Pucciniaceae family, *Uromyces vignae* and *U. appendiculatus*, as well as the species *Puccinia coronata* f. sp. *avenae* and *P. hordei*, formed a sister clade between each group, with a bootstrap value of 100. Nevertheless, *Tranzschelia discolor* showed a closer evolutionary relationship with *U. vignae*, *U. appendiculatus*, *P. coronata* f. sp. *avenae*, and with *P. hordei* than with *P. meibomiae* and *P.*

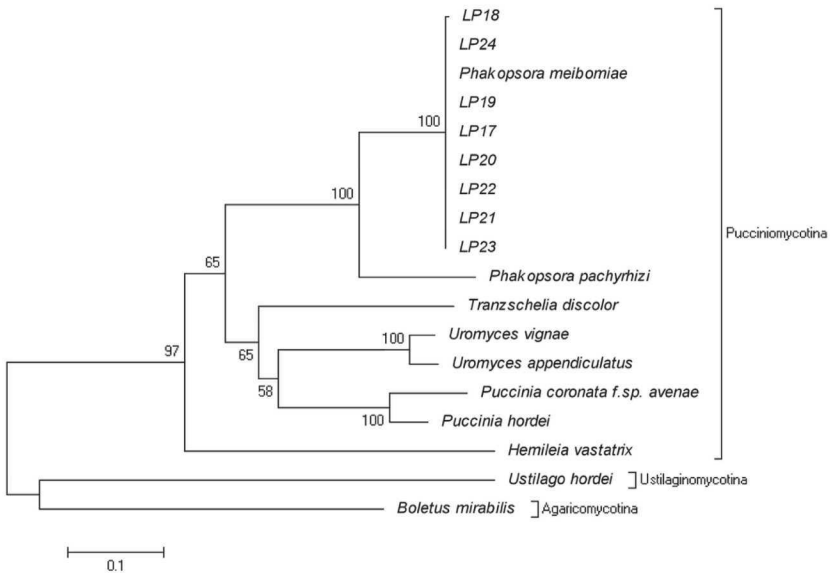


FIGURE 2. A Neighbor-Joining tree constructed from sequences of the ITS1-5.8S-ITS2 region, fungal species names and isolate numbers are shown in the figure; the bootstrap values calculated from 1,000 replicates are given above and below the branches.

pachyrhizi. Even though *Hemileia vastatrix* belongs to Pucciniomycotina subphylum, it showed a tight evolutionary relationship with species of the families Phakopsoraceae, Pucciniaceae, and Uropyxidaceae (*T. discolor*). Furthermore, *Ustilago hordei* (Subphylum: Ustilaginomycotina) and *Boletus mirabilis* (Subphylum: Agaricomycotina) showed a closer relationship than species belonging to the Pucciniomycotina subphylum (Cummins and Hiratsuka, 2004; Hibbett et al., 2007).

In summary, the findings presented here showed that the isolates collected on *L. purpureus* in the central mountain region of Puerto Rico correspond to *P. meibomiae*. Extensive surveys for genetic variation of *P. meibomiae* infecting wild legumes are needed to identify the possible source of Puerto Rican populations.

LITERATURE CITED

- Arthur, J. C., 1915. Uredinales of Porto Rico based on collections by F. L. Stevens. *Mycologia* 7: 315-332.
- Arthur, J. C., 1917. Uredinales of Porto Rico based on collections by H. H. Whetzel and E. W. Olive. *Mycologia* 9: 55-104.
- Bonde, M. R., S. E. Nester, C. N. Austin, C. L. Stone and R. D. Frederick, 2006. Evaluation of virulence of *Phakopsora pachyrhizi* and *P. meibomiae* isolates. *Plant Dis.* 90: 709-716.
- Bromfield, K. R., 1984. Soybean rust, Monograph No. 11. The American Phytopathological Society, St. Paul, Minnesota. 57 pp.
- Chatasiri, S., O. Kitade and Y. Ono, 2006. Phylogenetic relationships among *Puccinia hemerocallidis*, *P. funkiae*, and *P. patriniae* (Uredinales). *Mycoscience* 47: 123-129.
- Chatasiri, S. and Y. Ono, 2008. Phylogeny and taxonomy of the Asian grapevine leaf rust fungus, *Phakopsora ewittii*, and its allies (Uredinales). *Mycoscience* 49: 66-74.
- Cummins, G. B., 1943. Description of tropical rusts-V. *Bull. Torrey Bot. Club* 70: 68-81.
- Cummins, G. B. and Y. Hiratsuka, 2004. Illustrated genera of rust fungi. Third edition. American Phytopathological Society, St. Paul, Minnesota, 240 pp.
- Fernández, J., 2005. Incidencia y caracterización: morfológica, patogénica y genética de *Alternaria* spp. en cultivos de cebolla del sur de Puerto Rico. Tesis M.S. Universidad de Puerto Rico, Mayagüez, P.R., 167 pp.
- Frederick, R. D., C. L. Synder, G. L. Peterson and M. R. Bonde, 2002. Polymerase chain reaction assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae*. *Phytopathology* 92: 217-227.
- Gibbs, R. A. and M. Cockerill, 1995. Sequencher, version 3.0. Ann Arbor: Gene Codes Corporation.
- Hartman G. L., M. R. Miles and R. D. Frederick, 2004. Epidemiology and spread of soybean rust. American Seed Trade Association Conference Proceedings 1-9.
- Hall, T. A., 1999. BioEdit: a user-friendly biological sequence alignment and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.
- Henson, J. M. and R. French, 1993. The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* 31: 81-109.
- Hibbett, D. S., M. Binder, J. F. Bischoff, M. Blackwell, P. F. Cannon, O. E. Eriksson, S. Huhndorf, T. James, P. M. Kirk, R. Lucking, H. Thorstein Lumbsch, F. Lutzoni, P. B. Matheny, D. J. McLaughlin, M. J. Powell, S. Redhead, C. L. Schoch, J. W. Spata-

- fora, J. A. Stalpers, R. Vilgalys, M. C. Aime, A. Aptroot, R. Bauer, D. Begerow, G. L. Benny, L. A. Castlebury, P. W. Crous, Y. Dai, W. Gams, D. M. Geiser, G. W. Griffith, C. Gueidan, D. L. Hawksworth, G. Hestmark, K. Hosaka, R. A. Humber, K. D. Hyde, J. E. Ironside, U. Koljalg, C. P. Kurtzman, K. Larsson, R. Lichtwardt, J. Longcore, J. Miadlikowska, A. Miller, J. Moncalmo, S. Mozley-Standridge, F. Oberwinkler, E. Parmasto, V. Reeb, J. D. Rogers, C. Roux, L. Ryvarden, J. P. Sampaio, A. Schübler, J. Sugiyama, R. G. Thorn, L. Tibell, W. A. Untereiner, C. Walker, Z. Wang, A. Weir, M. Weiss, M. M. White, K. Winka, Y. Yao and N. Zhang, 2007. A higher-level phylogenetic classification of the fungi. *Mycol. Res.* 111: 509-547.
- Lamour, K., L. Finley, K. Snover, J. Stack, R. Hammerschmidt, J. Jacobs, J. Byrne, P. Harmon, A. Vitoreli, G. Wisler, C. Harmon, L. Levy, K. Zeller, C. Stone, D. Luster and R. Frederick, 2006. Early detection of Asian soybean rust using PCR. Online. *Plant Health Progress* doi: 10.1094/PHP-2006-0524-01-RS.
- Ono, Y., P. Buriticá and J. F. Hennen, 1992. Delimitation of *Phakopsora*, *Physopella* and *Cerotelium* and their species on leguminosae. *Mycol. Res.* 96: 825-850.
- Pan, Z., X. B. Yang, S. Pivonia, L. Xue and R. Pasken, 2006. Long-term prediction of soybean rust entry into the continental United States. *Plant Dis.* 90:840-846.
- Pfunder, M., S. Schürch and B. A. Roy, 2001. Sequence variation and geographic distribution of pseudoflower-forming rust fungi (*Uromyces pisi* s. lat.) on *Euphorbia cyparissias*. *Mycol. Res.* 105: 57-66.
- Pivonia, S. and X. B. Yang, 2004. Assessment of the potential year-round establishment of soybean rust throughout the world. *Plant Dis.* 88:523-529.
- Posada-Buitrago, M. L. and R. D. Frederick, 2005. Expressed sequence tag analysis of the soybean rust pathogen *Phakopsora pachyrhizi*. *Fungal Genetics and Biology* 42: 949-962.
- Rossi, R. L., 2003. First Report of *Phakopsora pachyrhizi*, the causal organism of soybean rust in the province of Misiones, Argentina. *Plant Dis.* 87: 102.
- Roy, B. A., D. R. Vogler, T. D. Bruns and T. M. Szaro, 1998. Cryptic species in the *Puccinia monoica* complex. *Mycologia* 90: 846-853.
- Schneider, R. W., C. A. Hollier, H. K. Whitam, M. E. Palm, J. M. McKemy, J. R. Hernandez, L. Levy and R. DeVries-Paterson, 2005. First report of soybean rust caused by *Phakopsora pachyrhizi* in the continental United States. *Plant Dis.* 89: 774.
- Smith, J. A., R. A. Blanchett and G. Newcombe, 2004. Molecular and morphological characterization of the willow rust fungus, *Melampsora epitea*, from arctic and temperate hosts in North America. *Mycologia* 96: 1330-1338.
- Szabo, L. J., 2006. Deciphering species complexes: *Puccinia andropogonis* and *Puccinia coronata*, examples of differing mode of speciation. *Mycoscience* 47: 130-136.
- Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.
- Vakili, N. G., 1981. Distribution of *Phakopsora pachyrhizi* on *Lablab purpureus* in Puerto Rico. *Plant Dis.* 65: 817-819.
- Vakili, N. G., 1979. Field survey of endemic leguminous hosts of *Phakopsora pachyrhizi* in Puerto Rico. *Plant Dis. Rep.* 63: 931-935.
- Vakili, N. G. and K. R. Bromfield, 1976. *Phakopsora* rust on soybean and other legumes in Puerto Rico. *Plant Dis. Rep.* 60: 995-999.
- Villavicencio, A. L., G. B. Fanaro, M. M. Araújo, S. Aquino, P. V. Silva and J. Mancini Filho, 2007. Detection of *Phakopsora pachyrhizi* by polymerase chain reaction (PCR) and use of germination test and DNA comet assay after e-beam processing in soybean. *Radiation Physics and Chemistry* 76: 1878-1881.
- White, T. J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of

fungal ribosomal RNA genes for phylogenetics. *In*: M. Innis, D. Gelfand, J. Sninsky and T. White (ed.). PCR Protocols a guide to methods and applications, pp. 315-322. Academic Press, San Diego, California.

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

