Special Report

Archaeophytopathology of *Phakopsora pachyrhizi*, the Soybean Rust Pathogen

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Abstract

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Herbarium specimens are useful to compare attributes of the past to attributes of today and predictions into the future. In this study, herbarium specimens from 1887 to 2006 were used to identify *Phakopsora pachyrhizi* and *P. meibomiae*, the two known fungal species that cause soybean rust. Historically, these two species differed in geographic distribution, with *P. pachyrhizi* confined to Asia and Australia, and *P. meibomiae* confined to the Americas. In our analyses, herbarium specimens were used to determine whether it was possible to extract adequate useful DNA from the fungal structures. If present, quantitative PCR primers specific to *P. pachyrhizi*, *P. meibomiae*, or to a third group inclusive of many rust species could be used to speciate the fungus. Of the 38 archival specimens, 11 were positive for *P. pachyrhizi*, including a 1912 specimen from Japan; 15 were positive for *P. meibomiae*, including a 1928 specimen from Brazil and two 1923 specimens from the Philippines; and 12 (including all African accessions) were negative for both species. Five specimens were positive in the more inclusive rust assay; all had been labeled as *P. pachyrhizi* and none were on soybean. These results demonstrate the feasibility of DNA genotyping in archaeophytopathological investigations.

Phakopsora pachyrhizi Syd. and *P. meibomiae* (Arthur) Arthur are two species of basidiomycete fungi that cause rust disease on a few leguminous crop plants, including soybean [*Glycine max* (L.) Merr.] and a number of other legume hosts belonging the subfamily Papilionoideae of the family Fabaceae (10,11), which is one of the largest families of flowering plants. These fungi are obligate pathogens of their hosts and have not been successfully cultured on any artificial medium. No alternate hosts have been reported for either *P. pachyrhizi* or *P. meibomiae* (8), and it is possible that a host supporting the aecial reproductive stage is either rare or extinct. Thus, the airborne repeating propagules (urediniospores) are the only means of dissemination, and no sexual recombination is available to facilitate genetic exchange. However, a recent report indicated that urediniospore germ tubes could fuse and their nuclei could migrate into the complex hyphal network, suggesting that nuclear exchange may be possible among isolates (13). Several species of *Phakopsora* have been detected in the United States, including *P. apoda*, *P. crotonis*, *P. gossypii*, *P. jatrophicola*, and *P. tecta* (2), but until the arrival of *P. pachyrhizi* to the continental United States in 2004, none causing disease on soybean were present. *P. meibomiae* is known to cause soybean rust in Mexico and the Caribbean (8), but has not been reported in the United States. *P. pachyrhizi* was well known in the Far East and Oceania, and is also reported in African locations, where it impacts present-day soybean production (7), although as recently as 1984, the African *Phakopsora* populations were suggested to be of a possibly different *forma specialis* (8).

Comparisons of DNA extracted from herbarium specimens have been widely used for analyzing genomes in many different kinds of biological samples (5), and more specifically to study fungal diversity in ancient documents (6) and to track historic migration of important plant pathogens like *Phytophthora infestans*, the cause of late blight of potato (9). In the case of soybean rust, herbarium specimens collected from many locations are available, some dating from the 1800s. Molecular diagnostic methods now exist (3) that employ quantitative PCR (qPCR) for the detection, differentiation, and quantification of the two species, and additional phylogenetic studies have utilized SSR fragment analysis (1,12) to make inferences on the genetic structure of what is essentially a clonal population of isolates. As a prelude to further molecular studies on the genetic structure of world populations of soybean rust using simple sequence repeat (SSR) markers (1), our objective was to use the herbarium specimens in our analyses to answer the simple question of whether it was possible to extract adequate useful DNA from the preserved fungal structures to perform species distribution studies over geographical space and time.

Materials and Methods

Thirty-eight samples of dried archival leaf tissue, which had been reported to contain *Phakopsora* spp., were sampled on location at the Arthur Herbarium at Purdue University, West Lafayette, Indiana (Table 1). All accessions were numerically identified along with the host genus (and usually species), and in most cases the year of collection was indicated along with a geographic location. The dried, pressed specimens had been stored at ambient herbarium temperature in the years (or decades) since collection in pouches of folded herbarium paper. Specimens were handled with clean forceps and examined under a dissecting microscope to locate uredinia characteristic of *Phakopsora*. Using a sterile scalpel, a segment of tissue 1 to 2 cm^2 containing uredinia was excised and placed in a sterile tube. Forceps were cleaned and gloves and scalpel blades were changed between herbarium accession pouches. Samples were transported to the Soybean Disease Laboratory at the University of Illinois.

All samples were photodocumented under a dissecting microscope (Fig. 1) taking equivalent care to preclude cross-contamination during handling, and then transferred to extraction tubes. DNA was extracted from the entirety of each sample using the FastDNA Spin kit with Lysing Matrix A and buffer CLS-VF with PPS (one of several extraction buffer options provided with the kit), as directed by the manufacturer (Qbiogene, Carlsbad, CA; now MP Biomedicals, Solon, OH). One microliter of sheared salmon sperm DNA (Applied

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Biosystems/Ambion, Austin, TX) at a concentration of 1 μg/μl was added to the extraction tube with the tissue to act as a carrier in anticipation of recovery of only trace quantities of nucleic acids. Tissue disruption occurred in the FastPrep FP-120 homogenizer (Bio 101-Thermo Electron Corp.; now MP Biomedicals, Solon, OH) for 40 s at a speed setting of 6 m/s^2. The aqueous eluate (100 μl) from the spun columns was diluted 50-fold with a diluent containing 5 mM Tris, pH 8, and 1 μg/ml salmon DNA. Five μl of the diluted DNA was assayed by multiplexed quantitative PCR (qPCR) in duplicate reactions.

qPCR assays used a Stratagene (Agilent Technologies, Santa Clara, CA) Mx3005p real-time thermal cycler and Invitrogen Platinum qPCR Supermix-UDG (Life Technologies Corp., Carlsbad, CA) in a 25 μl reaction volume. Reactions provided a final Mg^{2+} concentration of 7 mM and included 50 nM ROX dye as a passive reference. Oligonucleotide PCR primers specific to *P. pachyrhizi* or *P. meibomiae*, and a fluorogenic 5′-exonuclease linear hydrolysis probe, were commercially synthesized (IDT, Coralville, IA) according to the multicopy 5.8S and ITS2 rDNA target sequences (Ppm1, Ppa2, and Pme2, FAM probe) previously published (3). The qPCR thermal profile consisted of an initial uracil deglycosylase (UDG) incubation at 60°C for 2 min, then a 95°C denaturation/activation incubation for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. The Mx3005p was programmed for an adaptive baseline and automatic cycle thresholds, based upon dRn. Fifty ymol (30 copies) of an exogenous control target (PpaIC or PmeIC) was coamplified in every tube, as a multiplex reaction using the coreIC-Cy5 probe, to validate any negative results for both assays, as previously described (4). The UDG component of the supermix prevented false-positive results from adventitious contamination of reagents by amplicon of previous reactions. We previously demonstrated single-spore detection sensitivity with the Ppa assay (unpublished). All laboratory disposables were DNA-, RNA-, DNase-, and RNase-free; pipet tips included aerosol barrier filters.

A reference DNA to create standard curves for absolute quantification was extracted (FastDNA Spin Kit, manufacturer's directions) from a suspension of freshly heat-killed (50°C for 12 h) *P. pachyrhizi* isolate FL07-1 urediniospores (counted in a hemocytometer), and serially diluted. DNA of *Puccinia polysora* (the fungus causing southern corn rust) was also extracted. Urediniospores of *P. polysora* were a generous gift of J.K. Pataky (Department of Crop Sciences, University of Illinois, Urbana). These standard curves ranged from 500 to 0.05 spore-equivalents, and typically gave a RSq of greater than 0.995 and an efficiency of over 95%. DNA of *P. meibomiae* was the generous gift of R. Frederick (USDA-ARS, Ft. Detrick, MD) and used at a concentration of 1 pg/μl.

An additional qPCR assay ("all-rust") was designed to be more inclusive of additional rust species, targeting the rRNA large subunit gene. To design the all-rust assay, DNA sequences from five *Phakopsora* species and two *Puccinia* species (Table 2) were aligned using the Clustal

Table 1. Herbarium specimens (Arthur Herbarium at Purdue University) used to determine if the sample was *Phakopsora pachyrhizi* or *P. meibomiae* based on quantitative PCR primers specific to *P. pachyrhizi*, *P. meibomiae*, or to a third group inclusive of many rust species

Accession	Label identity	Year	Location	Host	Diagnosis
F12125	*P. pachyrhizi*	1931	Ishikawa, Japan	*Glycine max*	*P. pachyrhizi*
N5555	*P. pachyrhizi*	2006	Indiana, USA	*G. max*	*P. pachyrhizi*
F18518	*P. pachyrhizi*	1971	Queensland, Australia	*G. max*	*P. pachyrhizi*
F14033	*P. pachyrhizi*	1932	Kiangsi Prov., China	*Pueraria*	*P. pachyrhizi*
N4663	*P. pachyrhizi*	1994	Hawaii, USA	*G. max*	*P. pachyrhizi*
F1167	*P. pachyrhizi*	1924	Mindanao, Philippines	*Pueraria*	*P. pachyrhizi*
66727[a]	*P. pachyrhizi*	1913	Taipei, Taiwan	*Pachyrhizus*	*P. pachyrhizi*
N4510	*P. pachyrhizi*	2006	Indiana, USA	*G. max*	*P. pachyrhizi*
N4662	*P. pachyrhizi*	1994	Hawaii, USA	*G. max*	*P. pachyrhizi*
F14028	*P. pachyrhizi*	1932	Kiangsi Prov., China	*G. max*	*P. pachyrhizi*
F1164	*P. pachyrhizi*	1912	Kawauye-mura, Japan	*G. max*	*P. pachyrhizi*
64283	*P. pachyrhizi*	1971	Jalisco, Mexico	*Desmodium*	*P. meibomiae*
88336	*P. pachyrhizi*	1983	Veracruz, Mexico	*Pachyrhizus*	*P. meibomiae*
N4795	*Phakopsora*	1983	Minas Gerais, Brazil	*G. max*	*P. meibomiae*
66693	*P. pachyrhizi*	1980	Minas Gerais, Brazil	*Macroptillium*	*P. meibomiae*
N4579	*Phakopsora*	1983	Minas Gerais, Brazil	*Glycine*	*P. meibomiae*
87670	*Phakopsora*	NP[d]	NP	*Macroptillium*	*P. meibomiae*
89658	*P. pachyrhizi*	1988	Minas Gerais, Brazil	*Phaseolus*	*P. meibomiae*
N4796	*Phakopsora*	1983	Minas Gerais, Brazil	*Glycine*	*P. meibomiae*
66715	*P. pachyrhizi*	1928	Sao Paulo, Brazil	*G. max*	*P. meibomiae*
87221	*Phakopsora*	1983	Minas Gerais, Brazil	*Phaseolus*	*P. meibomiae*
F1174	*P. pachyrhizi*	1923	Lingayen, Philippines	*Crotalaria*	*P. meibomiae*
F1181[b]	*P. pachyrhizi*	1923	Mt. Arayat, Philippines	*Derris*	*P. meibomiae*
66707	*P. pachyrhizi*	1978	Sao Paulo, Brazil	*Neonotonia*	*P. meibomiae*
87293	*P. pachyrhizi*	1983	Sao Paulo, Brazil	*Neonotonia*	*P. meibomiae*
N4309	*P. pachyrhizi*	1986	Brazil	*Dolichos*	*P. meibomiae*
66231	*P. meibomiae*	1980	Veracruz, Mexico	*Erythrina*	Negative
F16214	*P. pachyrhizi*	1949	Sierra Leone	*Erythrina*	Negative
F16212	*P. pachyrhizi*	1957	Ghana	*Vigna*	Negative
F14027	*P. pachyrhizi*	1931	Anhwei Prov., China	*G. max*	Negative
90151	*Phakopsora*	1986	Minas Gerais, Brazil	*Eriosema*	Negative
89763	*P. pachyrhizi*	1887	Tonkin, Vietnam	*Pachyrhizus*	Negative
N3870	*P. pachyrhizi*	1979	Tanzania	*Vigna*	Other
3239[c]	*P. pachyrhizi*	NP	Isle of São Tomé, Africa	*Vigna*	Other
3240	*Phakopsora*	1917	Herradura, Cuba	*Teramnus*	Other
14035	*P. pachyrhizi*	1933	Kwangsi Prov, China	*Shuteria*	Other
66900	*P. pachyrhizi*	1982	Nsukka, Nigeria	*Erythrina*	Other
F16211	*P. pachyrhizi*	1946	Sierra Leone	*Vigna*	Other

[a] Type of *P. pachyrhizi* Sydow (http://plants.jstor.org/specimen/pur004212?s=t)
[b] Type of *P. calothea* Syd. (http://plants.jstor.org/specimen/pur004213?s=t)
[c] Type of *Uredo vignae* Bres. (http://plants.jstor.org/specimen/pur004211?s=t)
[d] Information was not available for the specimen.

W algorithm, and conserved sequences were identified and tested for functionality and selectivity, and a suitable set chosen which amplified *P. pachyrhizi*, *P. meibomiae*, and *P. polysora*, but neither salmon nor soybean DNA. The two primers were: ARF1 (5′-GTTGTTTGGGAATG CAGC-3′) and ARR3 (5′-CATCTTTCCCTCACGGTA-3′) and both were used at a final, optimized concentration of 300 nM in the assay. The probe was ARP1, synthesized (IDT) with a FAM reporter and an Iowa Black quencher (5′-FAM-CAAAGTGGGTGGTAAATTCCATC

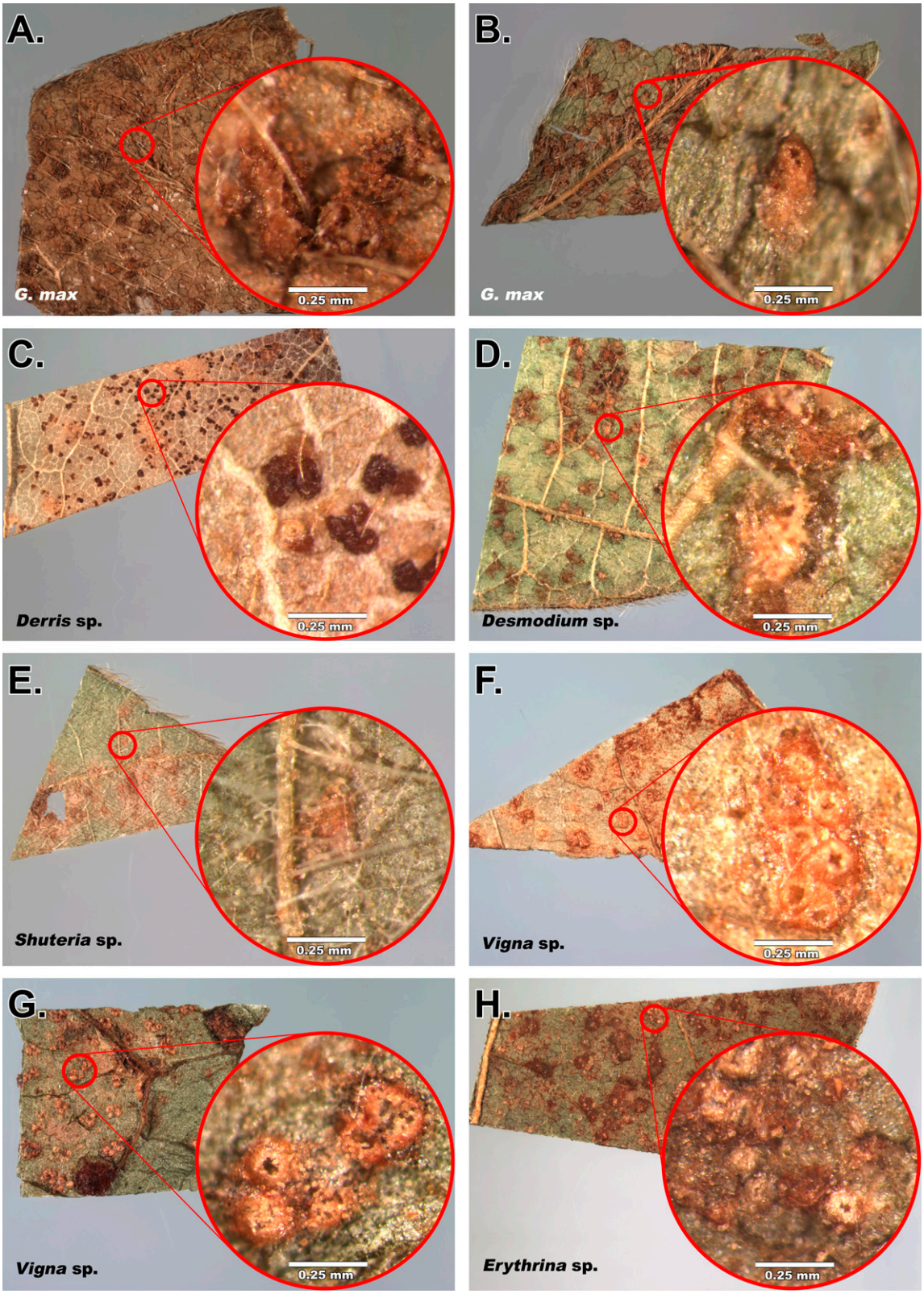

Fig. 1. Selected leaf samples (Arthur Herbarium at Purdue University = PUR) from which DNA was extracted; host plant as indicated; scale bar = 250 μm in the inset photo. All specimens had evidence of rust infection, including uredinia and residual urediniospores. A and B were found to contain DNA from *P. pachyrhizi*. **A,** Japan (1912; PUR accession F1164); **B,** Hawaii (1994; PUR accession N4662). C and D were found to contain DNA from *P. meibomiae*. **C,** Philippines (1923; PUR accession F1181); **D,** Mexico (1971). E, F, G, and H were found to be positive for the presence of a rust, but were negative for both *P. pachyrhizi and P. meibomiae*. **E,** China (1933; PUR accession 14035); **F,** Tanzania (1979; PUR accession N3870); **G,** Sierra Leone (1946; PUR accession F16211); **H,** Nigeria (1982; PUR accession 66900).

TAAGGC-IAB-3′), which was used at a final, optimized concentration of 100 nM in the assay with 5 mM Mg^{2+}. Primers and HEX-labeled probe for the HHIC multiplexed exogenous control assay, along with 50 ymol of linearized pJSH-B14 plasmid target (plasmid no. 20145 at addgene.org), were included in each reaction to validate negative results (4).

Results

DNA of *P. pachyrhizi* was successfully obtained and quantified by qPCR from many archival tissues, some quite old, including a specimen of soybean collected in Japan in 1912 (Fig. 1A). The yield of *P. pachyrhizi* DNA obtained from each accession is shown in Table 3, and the highest yield was from a 1913 Taiwanese accession, obtaining over 6,000 urediniospore-equivalents (SEq) of DNA. We have determined that 2 to 3 SEq of DNA is the minimum necessary for each of our SSR analyses (unpublished) and thus, to run four primer pairs in duplicate, approximately 25 SEq of DNA is required for each of our intended SSR analyses. Several of these archival specimens did, in fact, yield sufficient DNA for SSR analyses, demonstrating the potential for archival specimens to facilitate molecular genetic studies. Although estimated as SEq of DNA, this assay does not discriminate between *P. pachyrhizi* DNA from mycelium, haustoria, urediniospores, or other fungal structures, nor do these assays discern living from dead cells. The diagnostic result of each specimen tested is shown in Table 1. Eleven of the 38 specimens were found to contain *P. pachyrhizi* and not *P. meibomiae*; they dated from 1912 to 2006. Fifteen specimens were found to contain *P. meibomiae* and not *P. pachyrhizi*; they dated from 1923 to 1988 (e.g., Fig. 1C and D). Half of the remaining 12 specimens, which were negative for both *P. pachyrhizi* and *P. meibomiae*, were found to amplify in the all-rust assay, indicating presence of rust DNA belonging to another species (Fig. 1E to H). These specimens were collected from Tanzania (1979), Nigeria (1982), São Tomé, China (1933), and Sierra Leone (1946), and none of them were from soybean. The final six specimens contained no amplifiable DNA and were inconclusive; they dated from 1887 to 1980. No *P. pachyrhizi* was observed in any specimen from Africa; however, *P. meibomiae* was determined in two 1923 specimens from the Philippines, and one unidentified rust was observed in the 1933 specimen from China. The positive and negative control reactions run with each experiment gave the expected positive or negative result (data not shown), and the multiplexed internal control reactions successfully validated all negative reactions, affirming the functionality of the reaction cocktail and the absence of inhibitors or mechanical, instrument, or analyst error.

Table 2. Rust species for which rRNA large subunit DNA sequences were aligned in the design of the all rust qPCR assay

GenBank Accession No.	Species
DQ354537	*Phakopsora pachyrhizi*
AB354766	*P. vitis*
AB354753	*P. euvitis*
AB354760	*P. meliosmae*
DQ354535	*P. tecta*
AY114289	*Puccinia graminis* f. sp. *tritici*
DQ460729	*P. striiformis* f. sp. *hordei*

Table 3. Yield of *Phakopsora pachyrhizi* DNA obtained, for those specimens (Arthur Herbarium at Purdue University) containing this species. Amounts are the estimated total amount of *P. pachyrhizi* DNA recovered from the entire specimen, expressed as urediniospore equivalents (SEq), corrected for dilution, to two significant figures

Accession No.	Yield (SEq)
66727	6,100
N4662	1,900
F12125	500
N4663	260
F14028	130
N4510	90
F1164	53
N5555	26
F18518	11
F1167	8
F14033	8

Discussion

Herbarium specimens dating from 1887 to 2006 were used to detect the soybean rust fungi, *P. pachyrhizi* and *P. meibomiae*. Of the 38 archival specimens, 11 were positive for *P. pachyrhizi*, including a 1912 specimen from Japan, but no *P. pachyrhizi* was found in specimens before 1994 outside of Asia or Australia, indicating that *P. pachyrhizi* was confined to the Eastern Hemisphere (8). Fifteen specimens were positive for *P. meibomiae*, including a 1928 specimen from Brazil and two 1923 specimens from the Philippines. This is the first report of *P. meibomiae* occurring outside the Western Hemisphere (8). Because the samples were destructively extracted, and because it is unlikely that the 85-year-old specimen material remaining in the herbarium would contain viable urediniospores, it is not possible to perform Koch's postulates to support this observation. However, sequencing or immunoassay tests beyond the scope of this manuscript could be attempted. The world distribution of *P. meibomiae* remains unknown, as well as its primary host. The species likely evolved with legumes, and its distribution is somewhat known in the Western Hemisphere, but nothing is known about its distribution elsewhere other than our finding it in herbarium specimens from the Philippines. Without further worldwide sampling, questions will remain about the hosts and distribution of *P. meibomiae* outside the Western Hemisphere. The answers may reveal potential genetic or cultivational solutions for disease management of both species.

Interestingly, there were six specimens found to contain some species of rust not being *P. pachyrhizi* or *P. meibomiae*, although uredinia were present (Fig. 1E to H) and historical examination had suggested a tentative identity of *P. pachyrhizi*. These may represent one or more *formae specialis*, or entirely separate species of *Phakopsora.* None of these were from *G. max*; three were on species of *Vigna*, and one each on *Erythrina*, *Teramnus*, and *Shuteria*. An alternative explanation could be that the available qPCR assays for *P. pachyrhizi* and *P. meibomiae* are not completely inclusive, as they were developed based upon sequence data from a limited number of isolates. Thus, one or both of the target species may not have been fully detected because of undetermined variation. The same complaint may be lodged against many molecular diagnostic assays for plant pathogens; however, those assays remain useful until anomalies are observed, at which point they can be improved. Whether such variation existed or still exists in *Phakopsora* spp. is unknown, and is a reasonable point of inquiry for future research. There were six more specimens that were negative in every assay and probably represent instances in which all DNA had degraded over time, as a result of preservation, harsh storage conditions during transportation, or other environmental factors, and include not only the sample collected on the eponymous *Pachyrhizus* species in 1887 in Vietnam, but also the sample collected almost a hundred years later on *Erythrina*, in 1980 in Veracruz, Mexico. There was no apparent correlation between the age of samples and the yield of rust DNA obtained in this experiment, as the highest yield came from a 1913 Taiwanese specimen, while the second-highest yield came from a 1994 Hawaiian sample (Table 3). However, note that no attempt was made to sample equivalent numbers of uredinia.

It is interesting to note that there was no overlap in the molecular identification of any of the specimens determined in this study to be of the *Phakopsora*; i.e., no specimen positive for *P. pachyrhizi* was also positive for *P. meibomiae* or vice versa. This could be of significance pathologically since it points to the fact that *P. pachyrhizi* and *P. meibomiae* may have moved or occurred beyond their initially defined geographical boundaries, and also from the perspective of sample integrity. Unknown and possibly considerable numbers of other workers over the last century have had the opportunity to examine and sample from these herbarium specimens, and certainly it has been possible that residue (urediniospores, tissue fragments, etc.) could have been inadvertently transferred between herbarium pouches. Although we did not examine any host specimens that

explicitly claimed not to be infected with rust, it would seem that we were not alone in exercising caution to prevent carryover from contaminating this valuable collection.

This study demonstrates that the identities of *P. pachyrhizi* and *P. meibomiae*, and possibly other rust species, are determinable by molecular genetic analysis of residue from dried host tissue specimens, some quite old, housed in herbaria without chemical or cryopreservation. Further, the DNA recovered can be of adequate quality for qPCR amplification and quantification, and in the amounts necessary for further genetic studies, such as SSR genotyping. While the geographic and temporal scope of this study was limited by the material available, the findings regarding the presence of *P. meibomiae* in the far east are novel; as additional archival materials become accessible, these results will be readily expanded. The careful curation of herbaria by past botanical scholars has created a legacy of unexpected utility for modern investigations that will help to further our understanding of fungal distribution and genetic diversity, which may be applied over time for practical benefit to agriculture and society.

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