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Genetic Diversity and Origins of *Phakopsora pachyrhizi* Isolates in the United States

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ABSTRACT

Soybean rust (SBR), caused by *Phakopsora pachyrhizi*, is a destructive disease worldwide. Recently it has emerged as a threat to soybean production in the United States (U.S.). There is limited knowledge regarding the genetic diversity and geographic distribution of *P. pachyrhizi* isolates in the United States. To address this issue, a survey was conducted to define the genetic diversity and possible origin(s) of U.S. isolates. The Internal Transcribed Spacer (ITS) sequence and the ADP-Ribosylation Factor (*ARF*) gene sequence were amplified and cloned from a collection of 59 isolates. Consequently, the cloned ITS and *ARF* sequences were subject to phylogenetic analyses. At least five genotypes were identified within the U.S. isolates and most states contained a mixture of isolates. Gauged by the DNA sequences derived from international and archival isolates, two groups of global isolates that are endemic to all continents were identified. This reinforces the hypothesis of a global migration of *P. pachyrhizi*. However, there are also two groups of isolates that appeared to be present only in Asia, Australia and U.S. This suggests the presence of a possible alternative migratory pathway. One isolate type appeared to be unique to the U.S., suggesting either a unique origin or rapid diversification of isolates collected from the U.S. The data provide the most comprehensive analysis to date of the genetic diversity of *P. pachyrhizi* isolates in the U.S.

Key words: *Phakopsora pachyrhizi*, soybean rust, genetic diversity, phylogeographic distribution, soybean breeding

INTRODUCTION

Soybean rust (SBR), caused by the fungal pathogen *Phakopsora pachyrhizi* Syd., is an economically important disease of soybean worldwide. Although, *P. pachyrhizi* can cause significant yield losses ranging from 5-95% in continents other than North America (Bromfield, 1984;

Hartman *et al*., 1991; Yang *et al*., 1991a; Yorinori *et al*., 2005), severe yield losses have not been observed in the U.S., likely due to unfavorable disease conditions. However, it is possible that potential high yield losses may occur in the future as the fungus continuously adapts within the U.S. and/or environmental conditions allow for favorable spread of the disease during the growing season.

Phakopsora pachyrhizi has been well documented in Asia since the 1900's and has subsequently been reported in Hawaii in 1994 (Killgore *et al*., 1994), in Southern and Central Africa from 1997-2001 (Levy, 2005) and in South America since 2001 (Yorinori *et al*., 2005; Freire *et al*., 2008). In 2004, *P. pachyrhizi* became a newly emerged threat to U.S. soybean production (Stokstad, 2004; Harmon *et al*., 2005; Schneider *et al*., 2005). The prevailing opinion is that *P. pachyrhizi* entered the U.S. through hurricane winds from a source in South America (Magarey and Isard, 2005; Schneider *et al*., 2005). However, this hypothesis lacks solid experimental support and, therefore, the means by which *P. pachyrhizi* urediniospores migrated globally and finally reached the U.S. remains undefined. Recently, computational modeling of spore dispersal, by integrating variable factors including weather conditions and alternative overwinter hosts, has become an interesting topic of *P. pachyrhizi* studies (Yang *et al*., 1991a, b; Levy, 2005; Yorinori *et al*., 2005; Del Ponte *et al*., 2006; Pan *et al*., 2006; Christiano and Scherm, 2007). However, these studies often focus on prediction of *P. pachyrhizi* epidemics in a specific geographic region or a single continent. To our knowledge, only one study sought to provide a dynamic model for intercontinental dispersal of *P. pachyrhizi* urediniospores (Pan *et al*., 2006). The recorded dates of occurrence of *P. pachyrhizi* in different locations supports the prevailing hypothesis that SBR originated in Asia, moved sequentially throughout Asia, to Australia and Africa and arrived in the U.S. from S. America.

The life cycle of *P. pachyrhizi* is microcyclic without a known sexual reproduction stage. Urediniospores and teliospores were the only two types of spores that have been observed from *P. pachyrhizi*. Teliospores have been observed in Asia on soybean hosts, but their germination has not been documented in soybean fields (Bromfield, 1984). However, teliospore germination and basidiospore production have been observed under laboratory conditions (Saksirirat and Hoppe, 1991). The dikaryotic urediniospore is the predominant form of *P. pachyrhizi* spore found throughout the growing season on soybean and an alternative host, kudzu. Typically, *P. pachyrhizi* produces a massive amount of dikaryotic urediniospores that are readily wind dispersed and multiple infection cycles can occur throughout a single growing season. Since, urediniospores are produced asexually and karyogamy and genetic recombination does not occur in this stage, urediniospore DNAs are expected to represent a single genetic unit. This makes the urediniospore stage an ideal target for studying *P. pachyrhizi* genetic diversity (Bromfield, 1984).

Deployment of *P. pachyrhizi*-resistant soybean cultivars is the preferred means of disease management. Six *P. pachyrhizi* resistance genes have been mapped to date, including *Rpp1* (Bromfield and Hartwig, 1980; McLean and Byth, 1980), *Rpp1b* (Ray *et al*., 2009), *Rpp2* (Hidayat and Somaatmadja, 1977), *Rpp3* (Hartwig, 1983), *Rpp4* (Hartwig, 1986; Meyer *et al*., 2009) and *Rpp5* (Garcia *et al*., 2008). However, commercially available cultivars that contain these *P. pachyrhizi* resistance genes are not currently grown in the U.S.

Despite the identification of resistance genes, monogenic soybean rust resistance has been shown to be labile and can be easily overcome by rust pathogens (Lin, 1966; Bromfield, 1984; Poonpolgul and Surin, 1985; Shin and Tschanz, 1986; Hartman *et al*., 2005). One recent study established a baseline of pathogenic variation of *P. pachyrhizi* in the United States

(Twizeyimana and Hartman, 2012). This study reported that there were only three pathotypes identified among the 72 U.S. *P. pachyrhizi* isolates based on the virulence of these isolates on soybean genotypes in a differential set. Our understanding of *P. pachyrhizi* adaptation is limited because of a lack of historic knowledge on the genetic diversity of *P. pachyrhizi* in soybean fields and on kudzu plants which serve as the overwintering host within the U.S. Therefore, there is a need to study genetic variation and the geographic distribution of genetic diversity of *P. pachyrhizi* isolates, with a specific interest on those *P. pachyrhizi* strains that are endemic to U.S. soybean production areas.

To define the genetic diversity of U.S. *P. pachyrhizi* isolates and their possible origin(s), Genetic diversity of isolates derived from across the soybean growing regions of the U.S. were examined in this study. These isolates were augmented by isolates collected from various international soybean production areas. The genetic diversity and geographic distribution of *P. pachyrhizi* isolates were determined by analyzing the DNA sequences of Internal Transcribed Spacer (ITS) and the ADP Ribosylation Factor (*ARF*) gene. Possible origins and migration paths of U.S. isolates are also discussed.

MATERIALS AND METHODS

Collection of SBR isolates: The majority of *P. pachyrhizi* isolates in this study were collected directly from soybean fields in major continents from 2001-2008 (Table 1). For environmental samples collected by the University of Missouri group, urediniospores were collected on site from the bottom of leaves using a pump-driven spore collector (Cyclone surface sampler, Burkard Manufacturing Co. Ltd, Rickmansworth, UK) and were germinated immediately for on-site genomic DNA extraction. The Nigerian samples from 2001 were directly collected from soybean fields and heat-killed before DNA extraction. Purified isolates provided by the Monsanto Company were maintained in a biosafety containment facility to allow repeated transfer as single pustules on leaves. For isolates from the FDWSRU collection at Ft. Detrick, MD, each isolate was originally received as infected leaf tissue or as urediniospores collected from the field. After being sent to the ARS containment laboratory at Ft. Detrick, each isolate was increased and maintained in greenhouses on soybean cv. Wayne or Williams.

Genomic DNA extraction from *P. pachyrhizi*: The extraction of *P. pachyrhizi* genomic DNA performed by the University of Missouri group utilized the CTAB method adapted from (Pitkin *et al*., 1996). Genomic DNAs from the North Carolina group were extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) from infected soybean leaves. The presence of only *P. pachyrhizi* sequences in the extract was verified by PCR amplification with the Expand High Fidelity Plus System from Roche (Mannheim, Germany) and primers specific for *P. pachyrhizi* [Ppa1/Ppa2] and *P. meibomiae* [Pme1/Pme2] (10). Genomic DNA from five Nigerian isolates was extracted from urediniospores (up to 30 mg of each isolate) by the University of Illinois group using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Genomic DNA from isolates from the FDWSRU collection at Ft. Detrick was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA).

PCR amplifications, cloning and sequencing: The targeted genes were the Internal Transcribed Spacer sequences (ITS) and other housekeeping genes encoding ADP-ribosylation factor (ARF, DN739857), polyubiquitin 5 (DN740148) and a putative 14-3-3 protein (DN39688).

Table 1: List of *Phakopsora pachyrhizi* isolates included in this study

Name	State/province	Country	Sources of isolates
AU79-1	Not known	Australia	FDWSRU collection
BZ01-1	Parana	Brazil	FDWSRU collection
BZ09-1	Londrina	Brazil	FDWSRU collection
CN03-1	Yunnan	China	FDWSRU collection
CN08-1	Hubei	China	The Missouri group
CO04-1	Caicedonia	Colombia	FDWSRU collection
IN73-1	Pantnagar	India	FDWSRU collection
NG05-1	Not known	Nigeria	The Illinois group
NG05-10	Not known	Nigeria	The Illinois group
NG05-34	Not known	Nigeria	The Illinois group
NG05-115	Not known	Nigeria	The Illinois group
NG05-17	Not known	Nigeria	The Illinois group
PG01-3	Not known	Paraguay	FDWSRU collection
PG08-1	Not known	Paraguay	FDWSRU collection
PH77-1	Not known	Philippines	FDWSRU collection
SA01-1	Not known	South Africa	FDWSRU collection
TH02-1	Not known	Thailand	FDWSRU collection
TW72-1	Taiwan	China	FDWSRU collection
TW80-2	Taiwan	China	FDWSRU collection
VT05-1	Not known	Vietnam	FDWSRU collection
VT08-4	Bac Giang	Vietnam	The Missouri group
VT08-5	Vinh Phuc	Vietnam	The Missouri group
VT08-7	Ha Tay	Vietnam	The Missouri group
VT08-1	Ha Noi	Vietnam	The Missouri group
VT08-2	Yen Bai	Vietnam	The Missouri group
ZM01-1	Turk Mine	Zimbabwe	FDWSRU collection
AL05-1	Alabama	United States	The Monsanto group
AR07-4	Arkansas	United States	The North Carolina group
AR07-2	Arkansas	United States	The North Carolina group
FL07-1	Florida	United States	The Illinois group
kFL08-1[a]	Florida	United States	The Missouri group
kFL08-2[a]	Florida	United States	The Missouri group
FL08-1	Florida	United States	The Missouri group
FL08-2	Florida	United States	The Missouri group
FL07-4	Florida	United States	The North Carolina group
GA08-1	Georgia	United States	The Missouri group
HW94-1	Hawaii	United States	FDWSRU collection
IL06-1	Illinois	United States	The North Carolina group
IL07-1	Illinois	United States	The North Carolina group
IA07-2	Iowa	United States	The North Carolina group
LA08-1	Louisiana	United States	The Missouri group
MS07-1	Mississippi	United States	The North Carolina group
MS07-2	Mississippi	United States	The North Carolina group
MS07-3	Mississippi	United States	The North Carolina group
MS07-4	Mississippi	United States	The North Carolina group
MS07-5	Mississippi	United States	The North Carolina group
MS07-6	Mississippi	United States	The North Carolina group
MS08-1	Mississippi	United States	The Missouri group

Table 1: Continue

Name	State/province	Country	Sources of isolates
MS08-2	Mississippi	United States	The Missouri group
MO06-1	Missouri	United States	The Monsanto group
NC06-1	North Carolina	United States	The North Carolina group
OK07-1	Oklahoma	United States	The North Carolina group
OK07-2	Oklahoma	United States	The North Carolina group
OK07-3	Oklahoma	United States	The North Carolina group
TN07-1	Tennessee	United States	The North Carolina group
TN07-2	Tennessee	United States	The North Carolina group
TX08-1	Texas	United States	The Monsanto group
kTX08-1[a]	Texas	United States	The Missouri group
TX07-1	Texas	United States	The North Carolina group

[a]*Phakopsora pachyrhizi* isolates were isolated from Kudzu plants

In our preliminary study, the *polyubiquitin 5* gene and *14-3-3* gene regions did not show extensive sequence polymorphisms. Therefore, ITS and the *ARF* gene sequences were the focus of the study.

The ITS sequence was amplified with a pair of primers (ITS4, 5'-TCCTCCGCTTATTGATATGCTT-3'; ITS5, 5'-GGAAGTAAAAGTCGTAACAAGG-3'). The *ARF* gene was amplified with a pair of primers (forward, 5'-CACCAACAACTTCAGTAGACAGTTT-3'; reverse, 5'-GAGCCATTCCAGGCCCTCGTACAA-3'). PCR amplifications were performed using a high fidelity polymerase Phusion (New England Biolabs, Ipswich, MA). An adenine tail was added to the Phusion PCR products using *Taq* DNA polymerase (Promega, Madison, WI) before ligating into pGEMT EZ vector (Promega, Madison, WI) following manufacturer's manual. After an overnight incubation at 4°C, the ligations were transformed into *Escherichia coli* grown on LB agar plates supplied with X-Gal. White colonies were subjected to colony PCR to identify the putative SBR clones using T7 primer and one of the sequence-specific primers.

Plasmid DNA was isolated following standard alkaline lysis protocols. Cloned SBR DNAs were further purified by precipitation with polyethylene glycol (PEG) prior to DNA sequencing. Briefly, 200 μL of the DNA solution was mixed with 100 μL 30% PEG, 30 mM $MgCl_2$ and then centrifuged at 10,000 xg for 15 min. The resulting DNA pellet was resuspended in 100 μL TE and then precipitated with ethanol and centrifuged again. The final pellet was resuspended in 50 μL of TE buffer, pH 8.0. Four to six individual clones for each locus per isolate were sequenced to ensure the maximum representation of genetic diversity. DNA sequencing was performed at the University of Missouri DNA Core facility (http://www.biotech.missouri.edu/dnacore/) using a 3730xl 96-capillary DNA Analyzer with Applied Biosystem Big Dye Terminator cycle sequencing chemistry. Sequencing results were analyzed using FinchTV developed by Geospiza (Seattle, WA).

Sequence analyses: The ITS1, the intervening 5.8S gene, ITS2 and part of the surrounding 18S and 26S gene sequences, amplified by ITS4 and ITS5, were used as one single input sequence (approximately 666 bp long) in our analyses. Totally, over 700 and 400 sequences were generated for the ITS and *ARF* gene, respectively. Duplicate sequences were removed from final analysis, leaving a total of 118 ITS sequences (GenBank accession number JN872907-JN873025) and 68 *ARF* sequences (GenBank accession number JN899167-JN89234) in our final analyses. Sequences were converted to a FASTA format, aligned using the Muscle program (Edgar, 2004) using default settings and viewed using JalView (Clamp *et al*., 2004) to identify the polymorphisms.

The aligned sequences were converted into Phylip format for calculating parsimonious phylogeny. The parsimony phylogenies were calculated using the "dnapars" program within the Phylip package (Felsenstein, 2000). The parameters are as following: U-Search for best tree, Yes; S-Search option, More thorough search; V-Number of trees to save, 10000; J-Randomize input order of sequences, Yes (seed = 3, 3 times); O-Outgroup root, No, use as outgroup species 1; W-Sites weighted, No; M-Analyze multiple data sets, Yes, 1000 data sets; I-Input sequences interleaved, Yes. The maximum-likelihood branch lengths were calculated using the Tree-Puzzle program (Schmidt *et al*., 2002). The parameters are as following: b-Type of analysis, Tree reconstruction; k-Tree search procedure, Evaluate user defined trees; m-Model of substitution, HKY; w-Model of rate heterogeneity, Gamma distributed rates; c-Number of Gamma rate categories, 4. The topologies were finally viewed using the ATV program (Zmasek and Eddy, 2001). For geographic distribution of *P. pachyrhizi* isolates, all isolate types were manually plotted geographically to display their frequency, spatial relationship and deduce the evolutionary origins and genetic diversity.

RESULTS

Twenty-nine polymorphisms, collectively from the ITS sequences and *ARF* gene sequences, were found from a total of 59 isolates examined (Table 1). The majority of these polymorphisms were Single Nucleotide Polymorphisms (SNPs) (17 total, 6 in coding regions and 11 in non-coding regions), followed by Indels (11 total, 2 in coding regions and 9 in non-coding regions). One microsatellite repeat sequence (in a coding region), a 6-bp CCCTCT repeat, was identified in the *ARF* gene. As expected, most of the environmental samples were a mixture of different isolates. Approximately 2.2 and 1.7 distinct alleles per environmental sample were identified based on ITS and *ARF* gene sequences, respectively. This is consistent with a previous report that the average number of alleles per locus on a list of 24 SBR loci examined was 3.9 (Anderson *et al*., 2008). One sample collected from Mississippi in 2007 (MS07-1) contained 9 distinct ITS sequences.

As reflected by the genetic diversity of ITS sequences, the U.S. isolates fall into at least five clades, including clades II, III, IV, VI and VII, in the parsimonious phylogenetic tree (Fig. 1), suggesting the presence of at least five groups of *P. pachyrhizi* isolates within the U.S. Each of these groups possesses a unique combination of polymorphisms. Geographically, each individual state harbors a mixture of *P. pachyrhizi* isolates ranging from four to nine, except for Alabama and Louisiana (Fig. 2a).

Similarly, a phylogenetic tree based on the *ARF* gene sequence reveals four *P. pachyrhizi* clades (clades I-IV) residing within the U.S. (Fig. 3). More importantly, the phylogenetic topologies based on the *ARF* gene and ITS sequences are partially overlapping (Fig. 1, 3). Specifically, isolates in clades II and IV of ITS phylogeny (ITS-II, ITS-IV) are also included in clade I of the *ARF* gene phylogeny (ARF-I). This also holds true for the matches of ITS-III versus ARF-II, ITS-VI versus ARF-III and ITS-VII versus ARF-IV (Fig. 1, 3). The geographic distribution map shows that each individual state harbors a mixture of isolates (Fig. 2c).

To further elucidate *P. pachyrhizi* genetic diversity and investigate the origins of U.S. isolates, the analyses were expanded to include global isolates and to compare domestic (U.S.) with international isolates. Included in the analyses is a group of recently collected international samples, as well as archival DNA samples previously collected from international locations. Clades VI and VII of the ITS phylogeny contain isolates from Asia, Africa, South America and the U.S.

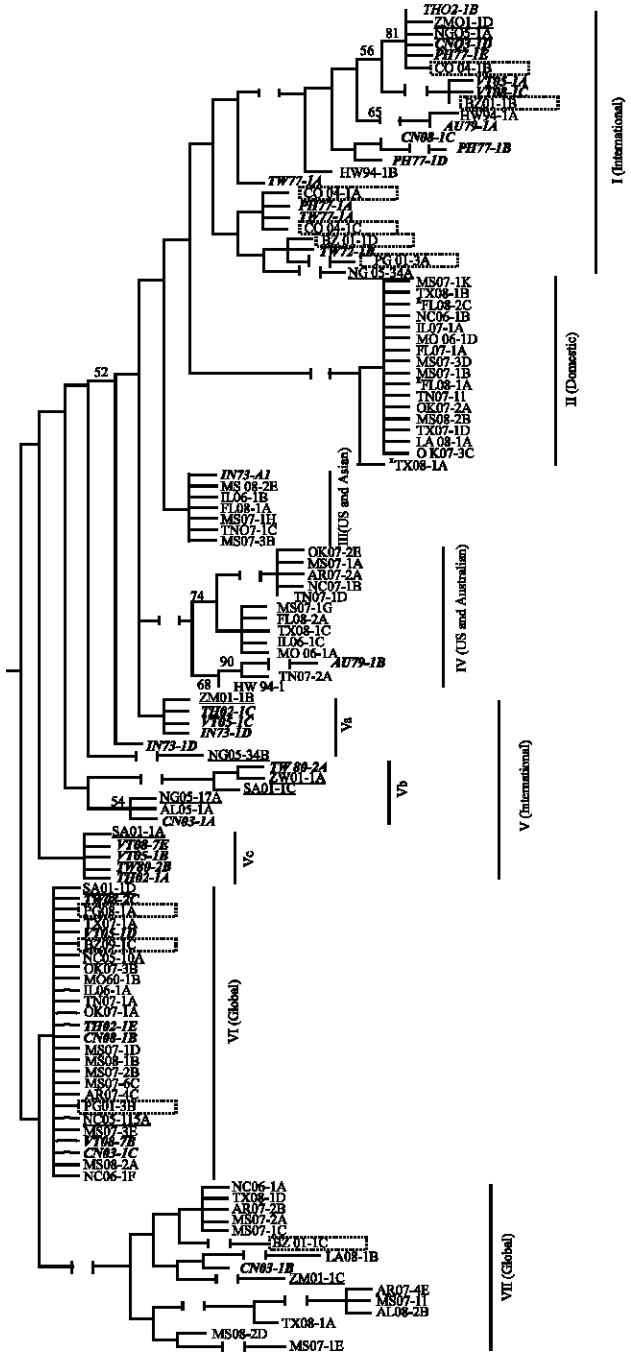

Fig. 1: Phylogenetic topology of soybean rust ITS sequences. A majority-ruled parsimony tree with maximum-likelihood branch lengths was calculated using a combination of ITS1, 5.8S gene and ITS2 sequences. Bootstrap values of 1,000 independent trees larger than 50 were labeled on each branch. Seven major clades are delimited by solid horizontal lines. A superscript "K" indicates those isolates were collected from kudzu plants. U.S. isolates are labeled regularly; Asian and Australian isolates are bold italicized; African isolates are underlined; and South American isolates are boxed. Country abbreviations for international isolates are included in Table 1

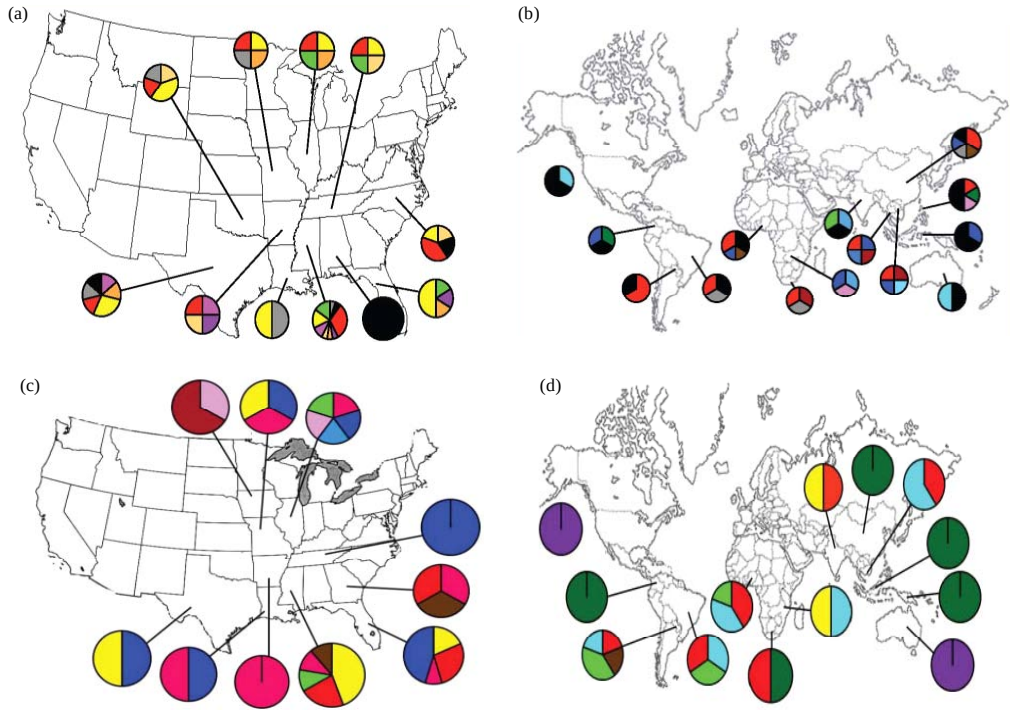

Fig. 2(a-d): Geographic distribution of SBR genetic diversity based on (a, b) ITS and (c, d) *ARF* sequences, Areas of pie charts represent frequency of isolate types

(Fig. 1). Similarly, clades III and IV of the *ARF* phylogeny are mixed isolates from all these continents (Fig. 3).

In the ITS phylogeny, besides the five clades containing domestic isolates, it was easy to identify two additional clades (clades I and V) that consist almost exclusively of international isolates, except for one Hawaiian isolate in clade I and one Alabama isolate in clade V (Fig. 1). Clade I contains a sample collected from Hawaii in 1994 (HW94-1). It is unlikely that this *P. pachyrhizi* isolate migrated to the continental U.S. Clade V contains a sample collected from Alabama in 2005, soon after the arrival of *P. pachyrhizi* in the U.S. in late 2004 (Harmon *et al*., 2005; Schneider *et al*., 2005). Similarly, clade V of the *ARF* phylogeny contains exclusively international isolates and shares isolates with that of clade I from the ITS phylogeny (Fig. 1, 3). Likely, these isolates are endemic on areas other than U.S. In contrast to those international isolates, a group of isolates, clade II of the ITS phylogeny and a subgroup within clade I of the *ARF* phylogeny, are exclusively domestic (Fig. 1, 3). In addition, the ITS clade II and a subgroup within *ARF* clade I are partially overlapping (Fig. 1, 3).

Clades III and IV of the ITS phylogeny contain mostly U.S. isolates and one Asian isolate and one Australian isolate, respectively (Fig. 1). Similarly, clade I and II of the *ARF* phylogeny contain mostly domestic isolates except for an Asian isolate and an Australian isolate, respectively (Fig. 3). These groups of isolates were not identified from Africa and South America within the scope of this study. Likely, these *P. pachyrhizi* isolates reside only within the continents of Asia, Australia and U.S. Interestingly, ITS clade IV and *ARF* clade I share the same Australian and Hawaii isolates, as well as some domestic isolates (Fig. 1,3). Sequence alignment shows that these isolates, compared to the remaining isolates, possess one additional repeat of a CCCTCT microsatellite motif.

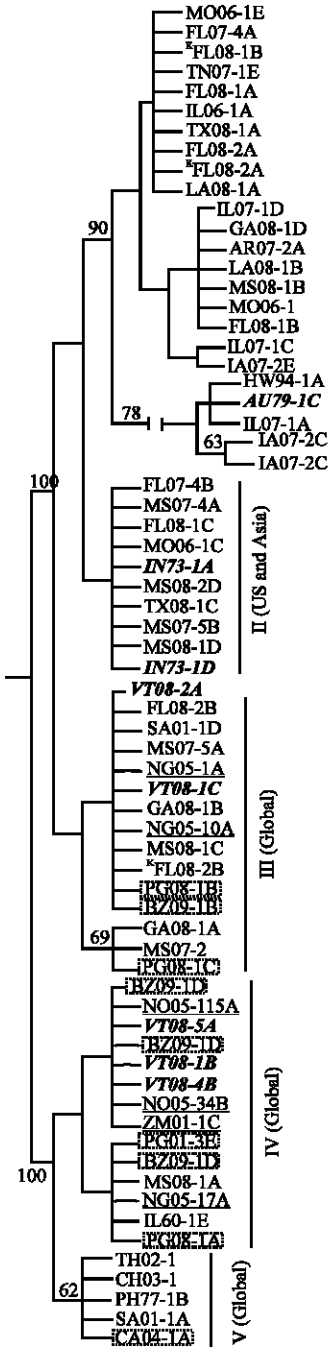

Fig. 3: Phylogenetic topology of rust *ARF* sequences. A majority-ruled parsimony tree with maximum-likelihood branch lengths was calculated using *ARF* sequences. Bootstrap values of 1,000 independent trees larger than 50 were labeled on each branch. Four major clades are delimited by solid horizontal lines. A superscript "K" indicates those isolates were collected from kudzu plants. U.S. isolates are labeled regularly; Asian and Australian isolates are bold italicized; African isolates are underlined; and South American isolates are boxed. Country abbreviations for international isolates are included in Table 1

DISCUSSION

This study utilized the DNA sequences to examine *P. pachyrhizi* genetic diversity. This approach reveals exact genetic polymorphisms, especially Single Nucleotide Polymorphisms (SNP), is suitable for optimizing phylogenic analyses and has the potential to identify specific molecular markers that could be used for strain detection and identification. Since, phylogeny based on ITS sequence may not be sufficient to distinguish each *P. pachyrhizi* isolate, a second gene, the *ARF* gene, was targeted to enhance the phylogenetic resolution of SBR isolates built from ITS sequences. Our results clearly show noticeable overlap between the phylogenies generated using ITS and *ARF* sequences, suggesting that combining multi-gene phylogenies can significantly reduce the bias resulted from a single-gene phylogeny, thus substantiating the robustness of our phylogenetic analyses.

Our results clearly demonstrate the presence of at least five types of isolates residing within the U.S. As might be expected, samples collected directly from soybean fields within the U.S., especially in Mississippi, Texas and Florida, were composed of a mixture of genotypes. Consistent with our results, a very recent study also reported a high level of genetic diversity of *P. pachyrhizi* in four agroecological zones in Nigeria (Twizeyimana *et al*., 2011). Phylogenetic analysis indicated the presence of five main groups of *P. pachyrhizi* and it was speculated that the high genetic diversity is likely due to the introduction of exotic and genetically distinct isolates (Twizeyimana *et al*., 2011). However, since rust urediniospores are dikaryotic, a single isolate can harbor two alleles for each unduplicated locus. Therefore, the predicted genetic diversity for each individual isolate from our ITS and *ARF* sequences may be over-estimated. However, this does not affect the geographic distributions deduced from the presence of specific sequences at different locations.

Based on the ITS phylogeny (Fig. 1), clade I contains isolates collected from continents other than U.S., except for isolates from Hawaii. It is probable that these isolates are endemic to other continents and have not reached the U.S. mainland or, at least, are not prominent there. Clade V contains one Alabama isolate (AL05-1) and mostly international isolates. However, it was unable to identify this isolate elsewhere within the U.S. during the period from 2006-2008. Given the fact that *P. pachyrhizi* urediniospores can be easily dispersed across a large area, it is not likely that this isolate was confined to a small area within Alabama. Presumably, this isolate did not adapt and was either lost or significantly reduced in prevalence.

Although, several globally distributed isolates were identified, there are a couple of isolates that appear to be present only in Asia, Australia and U.S., but not Africa and South America. These isolates are included in clades III and IV of the ITS phylogeny (Fig. 1) and clades I and II of the *ARF* phylogeny (Fig. 3). These data raise the possibility that some *P. pachyrhizi* isolates arrived in the U.S. from Asia/Australia via Hawaii. Alternatively, this isolate migrated in parallel from an unknown region (likely Asia) to both Australia and Hawaii followed by direct migration to U.S. via either Australia or Hawaii. However, it would require a much larger collection of domestic and international strains to directly examine this question further.

Interestingly, a group of isolates, ITS clade II (Fig. 1) and a subgroup of *ARF* clade I (Fig. 3), reside only in U.S. This finding raises the interesting possibility that some *P. pachyrhizi* strains may have been in the U.S., undetected, for quite some time. However, since the genetics of *P. pachyrhizi* are largely unstudied, the possibility of rapid genetic diversification accelerated by somatic hybridization (Vittal *et al*., 2011) or sexual reproduction cannot be ruled out.

Although, the current study examined a relatively large collection of *P. pachyrhizi* samples isolated over a period of several decades, it is likely that this collection does not represent the

complete genetic diversity of *P. pachyrhizi* isolates. Specifically, there are isolates that are domestic (Fig. 1, clade II-ITS; Fig. 3, clade I-ARF), isolates residing only in Asia and U.S. (Fig. 1, clade III-ITS; Fig. 3, clade II-ARF) and isolates residing only in Australia and U.S. (Fig. 1, clade IV-ITS; Fig. 3, clade I-ARF). It is quite possible that further sampling would reveal isolates that fall within these groups in other global soybean production areas. However, it would appear that these isolates are not currently prevalent in these areas.

Several specific resistance genes to *P. pachyrhizi* have been identified (Bromfield and Hartwig, 1980; McLean and Byth, 1980; Hartwig, 1983; Hartwig, 1986; Monteros *et al*., 2007; Garcia *et al*., 2008; Meyer *et al*., 2009). However, these resistance genes are only effective against selected *P. pachyrhizi* isolates (Lin, 1966; Bromfield, 1984; Poonpolgul and Surin, 1985; Shin and Tschanz, 1986; Hartman *et al*., 2005). Indeed, none of the cultivars currently planted in U.S. have durable field resistance to *P. pachyrhizi*, perhaps due to the large genetic diversity of domestic *P. pachyrhizi* isolates (Hartman *et al*., 2005). This diversity should be taken into account in future efforts to breed domestic soybean varieties for *P. pachyrhizi* resistance.

Current *P. pachyrhizi* detection methods rely on polymerase chain reaction amplification of ITS sequences (Frederick *et al*., 2002) or antibody-based recognition of *P. pachyrhizi* surface proteins (Mendes *et al*., 2009). However, on a practical level, these methods are unable to distinguish the full diversity of individual *P. pachyrhizi* isolates. The DNA polymorphisms identified in this study may be useful to further improve current diagnostic methods. Combining a measurement of *P. pachyrhizi* diversity with soybean breeding efforts is likely the most effective means to ultimately derive genotypes with durable *P. pachyrhizi* resistance for U.S. soybean production.

CONCLUSIONS

P. pachyrhizi is an important foliar disease of soybean, causing severe yield loss under favorable disease conditions. However, relatively little attention was paid to characterizing the genetic diversity of either domestic or international *P. pachyrhizi* isolates. In this study, a large scale phylogenetic analyses of soybean rust isolates, especially isolated collected from the U.S., was performed to examine the genetic diversity and possible origin of U.S. isolates. The results show that at least five genotypes were identified within the U.S. isolates and most states contained a mixture of isolates. Two groups of global isolates that are endemic to all continents were identified. However, there are also two groups of isolates that appeared to be present only in Asia, Australia and U.S. One isolate type appeared to be unique to the U.S. These findings not only reinforce the hypothesis of a global migration of *P. pachyrhizi*, but also suggest a unique origin or rapid diversification of isolates collected from the U.S. The data provide the most comprehensive analysis to date of the genetic diversity of *P. pachyrhizi* isolates.

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