Cercospora cf. *flagellaris* and *Cercospora* cf. *sigesbeckiae* Are Associated with Cercospora Leaf Blight and Purple Seed Stain on Soybean in North America

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Accepted for publication 10 May 2016.

ABSTRACT

Albu, S., Schneider, R. W., Price, P. P., and Doyle, V. P. 2016. *Cercospora* cf. *flagellaris* and *Cercospora* cf. *sigesbeckiae* are associated with Cercospora leaf blight and purple seed stain on soybean in North America. *Phytopathology* 106:1376-1385.

Cercospora kikuchii has long been considered the causal agent of Cercospora leaf blight (CLB) and purple seed stain (PSS) on soybean, but a recent study found *C. cf. flagellaris* associated with CLB and PSS in Arkansas (United States) and Argentina. Here, we provide a broader perspective on the distribution of *C. cf. flagellaris* on soybean and alternate hosts within the United States (Arkansas, Louisiana, Mississippi, Missouri, and Kansas). We used a multilocus phylogenetic approach with data from actin, calmodulin, translation elongation factor 1- α , histone 3,

the internal transcribed spacer region of rDNA and the mating-type locus to determine that two species, *C. cf. flagellaris* (200 of 205 isolates) and *C. cf. sigesbeckiae* (five of 205 isolates), are associated with CLB and PSS in the United States. In our phylogenetic analyses, species-level lineages were generally well-supported, though deeper-level evolutionary relationships remained unresolved, indicating that these genes do not possess sufficient phylogenetic signal to resolve the evolutionary history of *Cercospora*. We also investigated the potential for sexual reproduction in *C. cf. flagellaris* in Louisiana by determining the frequency of MAT1-1/MAT1-2 mating-type idiomorphs within the Louisiana population of *C. cf. flagellaris*. Though the MAT 1-2 idiomorph was significantly more common in our collection, the presence of both mating types suggests the potential for sexual reproduction exists.

Cercospora leaf blight (CLB) and purple seed stain (PSS) are diseases of soybean (*Glycine max* [L.] Merr) believed to be caused by *Cercospora kikuchii* (Mat and Tom [Gard]). CLB symptoms appear as purplish bronzing of leaves at the beginning of seed set. Necrotic lesions develop on the upper foliage of the canopy and later progress to the lower leaves, stems, and petioles (Walters 1980). Until around 2000, CLB was considered a minor disease in the Gulf South. However, it has since occurred more frequently (Cai et al. 2009), and yield losses have been reported from other soybean-growing regions in the United States (Geisler 2013; Hershman 2009) and South America (Almeida et al. 2006; Wrather et al. 2010). *Cercospora kikuchii* also infects soybean seeds, leading to characteristic symptoms that appear as irregular purple to pink areas of discoloration that vary in size from small blotches to the entire seed. Favorable climatic conditions, such as high atmospheric humidity and warm temperatures, during pod development promote the spread of PSS, which occurs wherever soybeans are grown (Chanda et al. 2014). Yield loss from PSS is considered negligible, though the disease can reduce seed quality (Jackson et al. 2006) and stunt or kill seedlings (Lehman 1950).

Though PSS was known to growers prior to its report by Suzuki (1921) in Korea, the association between *Cercospora* and PSS was first noted by R. Kikuchi in 1922. However, PSS was not considered a significant disease and received little attention (Matsumoto and Tomoyasu 1925). The first report of PSS from

the United States came from Indiana (Gardner 1926), followed by several other states in the eastern part of the country (Lehman 1928, 1950). A small percentage of seeds were initially observed to be affected (Lehman 1950), but as the disease presumably spread across many soybean-growing regions, symptomatic seeds were found with higher frequency (Schuh 1992).

Matsumoto and Tomoyasu (1925) first isolated and described *C. kikuchii* from purple-stained seeds and also reported that the fungus caused irregular purple-red leaf spots or lesions on leaves, pods, and stems. Similar symptoms were later reported from Taiwan (Han 1959) and the United States (Murakishi 1951; Walters 1980). Similarities between cultural and morphological characteristics of *C. kikuchii* and the fungus found to cause similar symptoms on soybean in North Carolina led Murakishi (1951) to conclude they were identical species.

A reliable and stable taxonomy for *Cercospora* has been hindered by the historical reliance on morphological characters, cercosporin production, and host association for species identification. Morphology has traditionally been used to identify *Cercospora* species, though this method may be inadequate or misleading in this and other genera, due to apparent homoplasy in the limited number of characters available for characterizing some species (Crous and Braun 2003; Goodwin et al. 2001; Groenewald et al. 2013; Herrera et al. 2015) and within-species variability that can be influenced by environmental conditions (Atkinson 1891; Horsfall 1929; Murakishi 1951). While some have also proposed cercosporin production may be of taxonomic value (Fajola 1978), cercosporin production is not a synapomorphy shared by all *Cercospora* species (Assante et al. 1977; Goodwin et al. 2001; Jenks et al. 1989), and our own observations indicate cercosporin production can vary within a species and even within a single isolate. Similarly, host specificity has proven unreliable for *Cercospora* taxonomy (Chupp 1954; Pollack 1987), with several species isolated from a broad

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

<http://dx.doi.org/10.1094/PHYTO-12-15-0332-R>
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range of hosts (Crous and Braun 2003; Groenewald et al. 2006, 2013) and others causing indistinguishable symptoms on the same host (Crous et al. 2006; Wang et al. 1998).

Since the description of *C. kikuchii* by Matsumoto and Tomoyasu in 1925, there have been few re-evaluations of this pathogen in a modern phylogenetic context. Several studies investigating genotypic and phenotypic variability within and among *C. kikuchii* populations (Cai and Schneider 2005, 2008; Imazaki et al. 2006; Latorre-Rapela et al. 2011) found differences among populations, but the identity of the fungus was based on host association. The internal transcribed spacer (ITS) rDNA region has been sequenced for many isolates collected from soybean and putatively identified as *C. kikuchii*, but nearly all these sequences share ≥ 99 percent similarity with many other *Cercospora* species deposited in GenBank. Goodwin et al. (2001) were able to show that *Cercospora* is monophyletic within *Mycosphaerella* (but see Bakhshi et al. [2015a]), however, isolates identified as *C. kikuchii* were polyphyletic based on phylogenetic analysis of ITS. These results highlight the need to re-evaluate the identity of *Cercospora* isolates previously considered *C. kikuchii*.

Groenewald et al. (2013) conducted a large-scale systematic study of more than 50 *Cercospora* species, establishing the monophyly of many species, using sequence data from five nuclear protein-coding genes and enabling a modern phylogenetic re-evaluation of *Cercospora* isolates previously assumed to be *C. kikuchii*. Soares et al. (2015) investigated the phylogenetic relationships among CLB and PSS-causing soybean pathogens, primarily in South America and Japan, and found that several lineages, including *C. cf. sigesbeckiae*, *C. kikuchii*, and *C. cf. flagellaris* were present in soybean-producing regions. They also sequenced few isolates from Arkansas, one of many soybean-producing states in North America, and found these to be *C. cf. flagellaris*. They speculated that the lineages associated with CLB and PSS are endemic to Asia and only recently radiated as a result of the unwitting dissemination of infected plant material following the proliferation of soybean as a commodity crop. However, based on relatively sparse sampling of soybean-growing regions throughout the Americas, particularly in North America, there is little evidence to strongly support any one hypothesis explaining the origin of these lineages.

The objective of this study was to test the hypothesis that multiple *Cercospora* species are associated with CLB and PSS in North America, two diseases long believed to be caused exclusively by *C. kikuchii*. To determine phylogenetic relationships of isolates identified as *C. kikuchii* collected from Louisiana and other soybean-producing states in the United States, we placed cercosporoid isolates from infected soybean seeds and leaves in the Gulf South and Midwest within a phylogenetic framework, using the five loci routinely utilized for systematic studies of *Cercospora*, and sequenced other cercosporoid fungi collected from two non-soybean host plants to investigate whether these were the same species found on soybean.

We also investigated the potential for cryptic sexual reproduction of *C. cf. flagellaris* in Louisiana. Cai (2004) previously identified these isolates as *C. kikuchii* and speculated that the high genetic diversity based on vegetative compatibility group pairings was the result of cryptic sexual recombination. Kim et al. (2013) found evidence for a cryptic sexual stage in Arkansas populations of *C. sojae*, another soybean pathogen, based on equal proportions of MAT1-1 and MAT1-2 idiomorphs. While the one or more pathogens causing CLB and PSS are widely believed to reproduce clonally, the results of Cai (2004) suggest sexual reproduction may be present. In order to directly address this hypothesis, we determined the frequency of the MAT1-1 and MAT1-2 idiomorphs among a collection of *C. cf. flagellaris* isolates from Louisiana to assess whether the potential for sex exists.

MATERIALS AND METHODS

Collection and Isolation. Cercosporoid fungal isolates were collected from soybean leaves and seeds displaying symptoms of

CLB and PSS and from leaf spots and localized necrotic lesions on *Gossypium hirsutum* and *Phytolacca americana*, respectively. Detailed information about all isolates used in this study is provided in Supplementary Table S1. Collection strategies, isolation techniques, and culturing methods were previously described (Cai 2004; Price 2013; Price et al. 2015) for all isolates included in this study, with the exception of those from foliar lesions on *P. americana* and *G. hirsutum*, and from soybean seeds originating in Arkansas, Kansas, Mississippi, and Missouri in the United States. Isolates collected from the leaves of *G. hirsutum* were obtained using the same technique as for soybean leaves (Price 2013). Leaf spots on *P. americana* were examined for cercosporoid fungi under a dissecting microscope. No conidia were observed, but fasciculate conidiophores resembling *Cercospora* were removed from lesions with a flamed glass needle and transferred to potato dextrose agar (PDA) (Becton Dickinson Difco, Sparks, MD, U.S.A.) amended with chloramphenicol (35 μg per liter). Soybean seeds and seed coats were dipped in 30% bleach solution, rinsed in three changes of distilled H_2O , and incubated on water agar. Isogenic cultures were established from single colony forming units (CFUs) by scraping a small amount of mycelium from the edge of a growing colony with a flamed needle, placing it in a 1.5-ml centrifuge tube filled with 1 ml of sterile Milli-Q (EMD Millipore, Billerica, MA, U.S.A.) molecular grade water, and vortexing. The suspension (50 μl) was dispersed onto a 100 $\times$ 15 mm petri plate containing PDA amended with chloramphenicol (35 μg per liter), using a flamed glass rod. Plates were incubated at room temperature and were inspected after 12 to 18 h, under a dissecting microscope, for the presence of hyphal growth. Individual CFUs were cut out of the agar with a sterile scalpel blade and transferred to 60 $\times$ 15 mm plates containing PDA.

DNA extraction. Isolates were grown on PDA amended with chloramphenicol (35 μg per liter) for 1 week prior to extraction of genomic DNA. A small amount of mycelium was scraped from the surface of the colony with a sterile scalpel blade, transferred to a glass test tube containing 10 ml of complete medium (Jenns et al. 1989), and incubated in a rotary shaker at 180 rpm for 3 to 4 days. Approximately 500 mg of wet tissue was transferred to a sterile 1.5-ml tube and was centrifuged at 14,000 rpm. Residual liquid was discarded and the remaining steps of the DNA extraction were performed as specified in the Promega Wizard DNA purification kit (Promega, Fitchburg, WI, U.S.A.). All samples were quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and diluted to a working concentration of 12.5 ng μl^{-1} .

Marker selection, polymerase chain reaction (PCR), and dataset assembly. The markers used in this study were chosen based on several recent phylogenetic studies of *Cercospora* (Crous et al. 2006; Groenewald et al. 2005, 2010, 2013). Primer sequences were obtained from the sources referenced below. Portions of actin (ACT), calmodulin (CAL), and translation elongation factor 1- α (EF-1 α) were amplified with the primer pairs ACT-512F/ACT-783, CAL-228F/CAL737R, and EF1-728F/EF1-983R (Carbone and Kohn 1999), respectively. Part of histone 3 (H3) was amplified with primers CYL H3F and CYL H3R (Crous et al. 2004) and *Cercospora*-specific mating type (MAT) genes were amplified with the primer pairs *Cercospora*Mat1f/*Cercospora*Mat1r and *Cercospora*Mat2f/*Cercospora*Mat2r (Groenewald et al. 2006). PCR cycling parameters for ACT, CAL, EF-1 α , H3, and MAT genes were also taken from the literature, though annealing temperatures were occasionally adjusted by several degrees for some isolates. ITS was amplified with the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), as previously described by Rush and Aime (2013).

PCR products were electrophoresed on 1% agarose gels at 120 V for 1 h and visualized under UV light to confirm target amplification. In the case of non-specific amplification, multiple bands were excised and purified using the Promega Wizard PCR and gel cleanup system (Promega). PCR products were direct-sequenced using Big Dye terminator chemistry on the Applied

Biosystems 3730xl platform at Beckman Coulter Genomics (Danvers, MA, U.S.A.).

Sequences were manually edited and contigs were assembled in Sequencher v5.0 (Gene Codes Corp., Ann Arbor, MI, U.S.A.). Generic identifications were made by comparing percent shared sequence identity of consensus sequences to others in the National Center for Biotechnology Information (NCBI) GenBank database with the blastn algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignments were estimated in MEGA6 (<http://www.megasoftware.net/>) (Tamura et al. 2013), using the Muscle (Edgar 2004) algorithm or MAFFT v7 (Katoh and Standley 2013), specifying a G-INS-I iterative refinement method and a 200PAM_k = 2 scoring matrix. Alignments were trimmed with GBlocks v0.91b (Castresana 2000; Talavera and Castresana 2007), allowing gap positions with smaller final blocks and less-strict flanking positions.

ACT, H3, and ITS (AHI) were consistently amplified in all isolates, but CAL and EF-1 α did not consistently amplify or were too weak to sequence, and nonspecific bands were frequently produced for CAL. Therefore, in order to facilitate choosing an appropriate subset of isolates for additional sequencing and phylogenetic analyses, we performed a haplotype analysis of the concatenated AHI alignment, including all CLB and PSS isolates and 14 additional isolates of *Cercospora* from *G. hirsutum* and *P. americana*. Each locus was first aligned individually and then concatenated into a single 1,069-bp alignment using DNAsp v5.10.1 (Librado and Rozas 2009). Using the same method, we also analyzed 67 MAT1-1 and 119 MAT1-2 sequence alignments

of 706 and 368 bp, respectively. The definition of a haplotype in this study is one or more sequences that are unique from all others based on at least one nucleotide polymorphism. We identified 30 AHI (multilocus), nine MAT1-1, and seven MAT1-2 haplotypes. Members of each haplotype are listed in Supplementary Table S2. CAL and EF-1 α were amplified and sequenced from one to three representatives of each multilocus haplotype. We estimated nucleotide alignments for three separate concatenated datasets, containing different combinations of sequences, to test genealogical concordance among individual gene trees and evaluate the impact of missing data on phylogenetic conclusions. The first dataset (DS-1) contained complete sequence information from ACT, CAL, EF-1 α , H3, and ITS of 15 CLB and PSS isolates representing 12 multilocus haplotypes and 186 additional reference sequences from 54 species of *Cercospora* generated by Groenewald et al. (2013). The second dataset (DS-2) was a supermatrix comprised of sequences of the same five loci from each representative of the 30 unique multilocus haplotypes plus 191 additional reference taxa representing 55 species of *Cercospora* generated by Groenewald et al. (2013). *Cladosporium herbarum* and *Cladosporium cf. subtilissimum* were used as outgroup taxa for DS-1 and DS-2. The third dataset (DS-3) contained the same loci as DS-1 and DS-2, but also included MAT1-1. The concatenated DS-3 alignment was a supermatrix consisting of nine CLB and PSS isolates with unique MAT1-1 haplotypes and also included 35 additional reference sequences from 14 *Cercospora* species, with *Mycosphaerella colombiensis* as an outgroup. Alignments and tree

Fig. 1. Maximum likelihood topology from RAxML analysis depicting the evolutionary relationships of 54 species of *Cercospora* based on a concatenated alignment of actin, calmodulin, translation elongation factor 1 α , histone 3, and internal transcribed spacer sequences (DS-1). *Cercospora kikuchii*, *Cercospora cf. flagellaris* and *Cercospora cf. sigesbeckiae* are shown in bold. Tree is rooted with *Cladosporium cf. subtilissimum* and *Cladosporium herbarum* as outgroup taxa. Asterisks indicate bootstrap percentages ≥ 70 and Bayesian posterior probabilities ≥ 0.95 . Clades A to G were collapsed for clarity and include the following taxa: (A) *C. senecionis-walkerii*, *C. coniogrammes*, *C. zeae-maydis* and *C. zeina*; (B) *C. apiicola*, *C. vignigena*, *C. euphorbiae-sieboldianae*, *C. punctiformis*, *C. achyranthis*, *C. polygonacea*, *C. campi-silii*, and *C. sojina*; (C) *C. pileicola*, *C. mercurialis*; (D) *C. olivascens*, *C. celosiae*, *C. physalidis*; (E) *C. dispori*, *C. chinensis*, *C. corchori*, *C. lactucae-sativae*, *C. cf. helianthicola*, *C. cf. citrulina*, *C. cf. nicotianae*, *C. cf. brunckii*, *C. apii*, *C. beticola*, and *C. cf. resedae*; (F) *C. chenopodii* and *C. cf. chenopodii*; (G) *C. agavicola* and *C. cf. coreopsidis*. The scale bar below the tree indicates the number of substitutions per site.

files are available in TreeBASE (submission ID 19128), and summary statistics for each alignment are provided in Supplementary Table S3.

Model testing, phylogenetic analysis, and species tree inference. The best-fit nucleotide substitution model for each partition was selected among a candidate set of 24 models according to corrected Akaike's information criterion (AICc) with MrModeltest v2.3 (Nylander 2004) for Bayesian inference (BI). The best-fit model was chosen among a candidate set of 88 models, according to AICc implemented in jModelTest 2 (Darriba et al. 2012; Guindon and Gascuel 2003) for maximum likelihood (ML) analysis in Garli v2.01 (Zwickl 2006). The following are the best-fit substitution models selected in MrModeltest/jModelTest for each partition: ACT – HKY + Γ /TPM3uf + I + Γ ; CAL – GTR + I + Γ /K80 + Γ ; EF1 – α – HKY + Γ /HKY + Γ ; H3 – HKY + Γ /JC; ITS – SYM + I/JC. Independent gene trees and the concatenated tree from a partitioned dataset were estimated in Garli v2.01, using the high-performance computing resources at Louisiana State University (www.hpc.lsu.edu/). Two separate analyses were run for each gene and the concatenated dataset. The starting tree for the ML search was generated by stepwise addition with 100 independent search replicates, ensuring that the best tree was found more than once. Each search replicate was terminated after 10,000 generations without an increase of more than 0.01 log-likelihood units. Bootstrap proportions were estimated from a minimum of 1,000 pseudoreplicate datasets, with the highest likelihood tree from two replicate searches per pseudoreplicate dataset retained. Bootstrap proportions were calculated and were mapped onto the ML phylogenetic trees, using SumTrees in the Dendropy v3.12.0 phylogenetic computing library (Sukumaran and Holder 2010). Additional ML analyses were done using the CIPRES portal (Miller et al. 2010). Both independent gene and concatenated RAXML trees were estimated using RAXML-HPC2 (Stamatakis 2006) on XSEDE v8.1.11 with 1,000 bootstrap replicates and specifying a GTR + Γ evolutionary model (raxmlHPC-HYBRID -n tre -s infile -x 12345 -N 1000 -k -p 12345 -f a -m GTRGAMMA). Bootstrap support values were estimated from 1,000 pseudoreplicate datasets. BI was performed for both independent genes and the partitioned, concatenated data, using MrBayes v3.2.3 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) on XSEDE via the CIPRES portal. Four replicates of 10

Fig. 2. Clade containing *Cercospora kikuchii*, *C. cf. sigesbeckiae*, and closely related species. The isolate shown in bold represents one multilocus haplotype shared by all of the five isolates of *C. cf. sigesbeckiae* from this study. Support values at nodes represent bootstrap percentages ≥ 70 (RAXML/Garli) and Bayesian posterior probabilities ≥ 0.90 (on right). An asterisk indicates a posterior probability of 1. The scale bar below the tree indicates the number of substitutions per site.

million generations each were run with four Metropolis-coupled chains, three heated to a temperature of 0.15. Trees were sampled every 1,000th generation with the first 25% discarded as burnin. Tracer v1.6.0 (Rambaut et al. 2014) was used to assess convergence of the estimated parameters. Graphical representations of phylograms were exported from FigTree v1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>) and edited in Inkscape v0.48.1 (<https://inkscape.org>).

Species trees were estimated from rooted gene trees under the coalescent method implemented in Maximum Pseudolikelihood for Estimating Species Trees (MP-EST) (Liu et al. 2010) and species tree estimation using average ranks of coalescences (STAR) (Liu et al. 2009). We assembled a complete dataset containing 54 *Cercospora* species and *Cladosporium herbarum* as an outgroup, using DS-1 as a template, but limited the number of sequences per taxon to three, which resulted in 117 terminals. Independent gene trees were estimated in RaxML, specifying 100 bootstrap replicates. These trees were then rooted with *Cladosporium herbarum*, using the species tree analysis web server (STRAW) (Shaw et al. 2013), and used to estimate the species tree with MP-EST and STAR in STRAW.

Characterization and statistical analysis of MAT loci. To test the null hypothesis that frequencies of MAT1-1 and MAT1-2 idiomorphs did not significantly deviate from a 1:1 ratio, a χ^2 goodness of fit test was performed using SAS v9.4 (SAS Institute Inc. Cary, NC, U.S.A.), assuming equal MAT frequencies. The sexual state of many species of *Cercospora* is not known, but it was

Fig. 3. Maximum likelihood topology from RAXML analysis depicting the evolutionary relationships of 14 species of *Cercospora* based on a concatenated alignment of actin, calmodulin, translation elongation factor 1 α , histone 3, internal transcribed spacer, and MAT1-1 sequences (DS-3). *Cercospora cf. flagellaris* isolates from this study are shown in bold. Tree is rooted with *Mycosphaerella colombiensis*. Support values at nodes represent bootstrap percentages ≥ 70 (RAXML/Garli) and Bayesian posterior probabilities ≥ 0.90 (on right). An asterisk indicates a bootstrap percentage of 100 or a posterior probability of 1. Double dashes indicate a bootstrap percentage < 70 or a posterior probability < 0.90 . The scale bar below the tree indicates the number of substitutions per site.

expected that relatively equal proportions of MAT1-1 and MAT1-2 idiomorphs would be indicative of sexually reproducing populations as in Kim et al. (2013). The MAT loci of 186 isolates of *C. cf. flagellaris* obtained from soybean leaves and seeds collected in Louisiana during 2000, 2011 and 2012 were characterized by a multiplex PCR assay using the primers *CercosporaMat1f/CercosporaMat1r* and *CercosporaMat2f/CercosporaMat2r* (Groenewald et al. 2006). Confirmation of each idiomorph was made based on PCR product size as visualized on a gel. MAT1-1 products were approximately 800 bp in length, while MAT1-2 products were approximately 450 bp.

RESULTS

Phylogenetic analyses. *Cercospora kikuchii* reference taxa, including sequences of the ex-type, formed a strongly supported monophyletic group in each of the concatenated analyses (DS-1, DS-2, DS-3) using both ML methods and BI, but none of the isolates collected during this study were nested within this clade (Figs. 1, 2 and 3). *Cercospora kikuchii* was weakly to moderately supported as monophyletic in the BI and ML independent gene analyses of ACT and CAL in DS-3 (results not shown), but was paraphyletic or polyphyletic in H3 and all independent gene tree analyses of DS-1 (Supplementary Figs. S1 to S5) and DS-2 (results not shown). The ML trees inferred in RAxML and Garli were largely consistent, therefore all ML results presented here and in supplementary figures are the inferences from RAxML.

All but five of the CLB/PSS isolates were distributed among 29 of the 30 multilocus haplotypes, nine MAT1-1 haplotypes and six of seven MAT1-2 haplotypes (Figs. 4 and 5). These isolates and 19 reference sequences of *C. cf. flagellaris* were monophyletic in all concatenated analyses (Figs. 1, 3, and 6) except for the Garli analysis of DS-1, where *C. cf. brunkii* was nested within a clade containing all isolates of *C. cf. flagellaris* (results not shown). *Cercospora cf. flagellaris* was also monophyletic in the CAL gene tree inferred from DS-1, DS-3 (Fig. 3) and in the RAxML analysis

of DS-2 (results not shown), but was paraphyletic or polyphyletic in all independent analyses of ACT, EF-1 α , H3 and ITS.

The five isolates that were not *C. cf. flagellaris* were all collected from symptomatic soybean leaves in four Louisiana parishes during 2012 and all belonged to multilocus haplotype 27 and MAT1-2 haplotype 5, neither of which were shared by any other isolates. These isolates nested within a monophyletic *C. cf. sigesbeckiae* in all concatenated analyses (Figs. 1, 2, and 3). *Cercospora cf. sigesbeckiae* was also weakly supported as monophyletic in all H3 gene trees, but was paraphyletic or polyphyletic in all other independent gene trees.

Species tree analyses. The topologies of the MP-EST (Fig. 7) and STAR species tree analyses were generally congruent with the concatenated analyses of DS-1, DS-2 and DS-3, though support values for interspecific and deeper relationships were generally weak to moderate. *Cercospora kikuchii*, *C. cf. sigesbeckiae* and *C. cf. richardiicola* were closely related, forming part of a lineage that also included *C. cf. malloti* and *C. rodmanii* in all analyses. *Cercospora kikuchii* was distantly related to *C. cf. flagellaris* in all species tree analyses and comprised part of a lineage that always included *C. cf. nicotianae* and twice contained *C. aff. canescens*.

Ratio of MAT1-1 and MAT1-2 idiomorphs. Among the 186 isolates of *C. cf. flagellaris* from Louisiana, there were 67 with the MAT1-1 idiomorph and 119 with the MAT1-2 idiomorph. There were no instances where an isolate possessed both MAT idiomorphs. The five isolates of *C. cf. sigesbeckiae* were all MAT1-2, but these were not included in the χ^2 test, which showed that there was a significant departure from the null hypothesis of an expected 1:1 ratio of MAT1-1 to MAT1-2 idiomorphs ($\chi^2 = 12.83$, $df = 1$, $P < 0.01$).

DISCUSSION

The assumption that *C. kikuchii* is the sole pathogen causing CLB and PSS on soybean globally has prevailed for many decades due to a paucity of distinguishing morphological characters to differentiate

Fig. 4. Frequency of AHI (ACT, H3 and ITS [internal transcribed spacer]) multilocus haplotypes observed for isolates of *Cercospora cf. flagellaris* and *C. cf. sigesbeckiae* analyzed during this study. Numbers along the x axis show the 30 haplotypes observed among 206 isolates of *C. cf. flagellaris* and *C. cf. sigesbeckiae* for which sequences of AHI were concatenated and analyzed as single gene fragments in the alignment. Haplotype 27 (asterisk) exclusively contained five isolates of *C. cf. sigesbeckiae*.

Fig. 5. Frequency of MAT1-1 and MAT1-2 haplotypes observed for isolates of *Cercospora cf. flagellaris* and *C. cf. sigesbeckiae* analyzed during this study. **A**, Nine MAT1-1 haplotypes were observed among 67 isolates of *C. cf. flagellaris*. **B**, Seven MAT1-2 haplotypes were observed among 119 isolates of *C. cf. flagellaris* and *C. cf. sigesbeckiae*. MAT1-2 haplotype 5 (asterisk) was unique to *C. cf. sigesbeckiae*.

species, persistent misconceptions about host specificity among *Cercospora* species and the lack of available sequence data representing a diversity of species within *Cercospora* to assess species assignments (Bakhshi et al. 2015a; Groenewald et al. 2013). In this study, we used multilocus and coalescent-based phylogenetic approaches to determine the identity of fungal pathogens causing CLB and PSS on soybean and to place these isolates in a broader evolutionary context. All of the cercosporoid isolates from symptomatic soybean leaves and seeds collected for this study were not nested within the *C. kikuchii* clade, indicating that *C. kikuchii* is not the cause of these diseases.

Two other species, *C. cf. flagellaris* and *C. cf. sigesbeckiae*, were associated with CLB and PSS. *Cercospora cf. flagellaris* represented all but five of the foliar isolates collected in Louisiana and all of the isolates collected from Arkansas, Kansas, Mississippi and Missouri. It was also isolated from leaf spots on *P. americana* collected in Arkansas, Illinois and Louisiana and on *G. hirsutum* in Louisiana. Given that leaf and seed isolates share multilocus haplotypes and that the *C. cf. flagellaris* clade in DS-1 contains both soybean leaf and seed isolates (Fig. 6), it appears that the same organism causes CLB and PSS. The predominance of *C. cf. flagellaris* on soybean in Louisiana and the fact that it was the only species detected in Arkansas, Mississippi, Missouri and Kansas suggests it is the primary species associated with CLB and PSS throughout the Gulf South and is present in other soybean-growing regions in the United States, including the Midwest.

Cercospora isolates cannot reliably be identified to species based on sequence similarity queries of GenBank using ACT, CAL, EF-1 α , H3, ITS, or MAT sequences. A nucleotide search of “*Cercospora kikuchii*” performed in the NCBI database on July 23, 2015 retrieved 159 hits (including two MAT1-1 and MAT1-2, four CAL, four H3, seven ACT, eight EF-1 α , and 33 ITS sequences). However, due to low levels of polymorphism, these loci are not very informative outside of a multilocus phylogenetic context, nor are they useful as barcodes, as also noted by Bakhshi et al. (2015b) and Groenewald et al. (2013). ITS is among the most frequently sequenced genes for studies of plant-pathogenic fungi, despite being inadequate both as a barcode locus and as a phylogenetic marker for many fungal lineages (Crouch et al. 2009; Hyde et al. 2009; Ko et al. 2011), including *Cercospora* (Bakhshi et al. 2015b; Groenewald et al. 2013). For example, the ITS sequence from the ex-type of *C. kikuchii* shares 100% identity with six other isolates of *C. kikuchii*, one isolate each of *C. chinensis*, *C. guatemalensis*, *Cercospora* sp. strain N, *C. tezpurenensis*, *C. zebrina*, and two isolates of *C. cf. richardiicola*. It also shares 99% identity with multiple isolates of >30 other *Cercospora* species. BLAST queries with the other loci sequenced for this study are similarly equivocal.

Phylogenetic analyses of all concatenated datasets were able to resolve some species within *Cercospora*. However, similar to the results of Bakhshi et al. (2015b) and Groenewald et al. (2013), the individual gene trees indicate an overall lack of phylogenetic signal for each locus, with few strongly supported monophyletic species. In the DS-1 gene tree analyses, similar to the observations of Groenewald et al. (2013), ITS was the least informative locus, where *C. zeae-maydis* and *C. zeina* were the only two taxa strongly supported by both ML and BI. We found that CAL and H3 gene trees contained the most bipartitions with bootstrap values ≥ 70 . This was partially in agreement with Groenewald et al. (2013), who reported that H3 and ACT provided the best species-level resolution, but also that no individual locus could distinguish among all species and that there were multiple instances of shared haplotypes among species. *Cercospora cf. flagellaris*, *C. kikuchii*, and *C. cf. sigesbeckiae* were among the species that Groenewald et al. (2013) found to be consistently problematic to distinguish. In our analyses of DS-1, *C. cf. flagellaris* and *C. cf. sigesbeckiae* were paraphyletic or polyphyletic in all gene trees except for CAL and H3, respectively, although support values were moderate to weak. *Cercospora kikuchii* was paraphyletic or polyphyletic in all independent gene trees.

Groenewald et al. (2013) found that *C. cf. flagellaris* was monophyletic and composed of two subclades in their concatenated five-gene analysis. The presence of these subclades, a wide geographic and host range, and the lack of morphological differentiation among subclades led them to propose that *C. cf. flagellaris* is likely a species complex. We also observed intraspecific variation within *C. cf. flagellaris*, though whether *C. cf. flagellaris* represents multiple cryptic species or a single widespread species remains in question. If *C. cf. flagellaris* is comprised of several cryptic species, future systematic studies may help to determine whether genetic diversity within this species correlates to geography, host range, or virulence. Recent phylogenetic analyses of other ubiquitous plant-pathogenic fungi, including *Colletotrichum gloeosporioides* (Doyle et al. 2013; Weir et al. 2012), *Colletotrichum acutatum* (Damm et al. 2012), and *Macrophomina phaseolina* (Sarr et al. 2014) have discovered cryptic species using some of the same loci commonly used for *Cercospora*. However, some of these markers provide less phylogenetic information in *Cercospora* than in other fungal genera, such as *Colletotrichum*.

Our results suggest that ACT, CAL, EF-1 α , H3, and ITS do not possess sufficient phylogenetic signal to resolve interspecific and deeper-level evolutionary relationships in *Cercospora*. ACT, CAL, EF-1 α , and H3 are orthologous, single-copy, protein-coding genes that are easily amplified in most *Cercospora* species. While these are typically the hallmark traits of good markers (Curto et al. 2012; Feau et al. 2011; Kepler and Rehner 2013), the regions within these genes historically targeted for phylogenetic analyses are relatively short (<400 bp) and conserved among closely related taxa. As a result, relationships between divergent taxa, such as *C. zeae-maydis* and *C. zeina*, are more strongly supported than those among

Fig. 6. Clade containing *Cercospora cf. flagellaris* isolates. Isolates from this study are shown in bold. Support values at nodes represent bootstrap percentages ≥ 70 (RAxML/Garli) and Bayesian posterior probabilities ≥ 0.90 (on right). Double dashes indicate a bootstrap percentage <70. Caret indicates bipartition not present in respective analysis. The scale bar below the tree indicates the number of substitutions per site.

The four *C. kikuchii* reference isolates were collected from Japan and Argentina. The three Japanese isolates, including the type, were isolated from *Glycine soja*, and the single Argentinian isolate from *G. max*. Though there are reports of *C. kikuchii* from other *Glycine* species, as well as other genera within Fabaceae, including *Cyamopsis*, *Dolichos*, *Phaseolus*, and *Vigna* (Farr and Rossman 2015), these isolates have not been evaluated in a phylogenetic context. *Cercospora kikuchii* is also not the only cercosporoid fungus reported from *Glycine*. Chupp (1954) reported 18 species associated with the plant genera listed above as well as the close *Glycine* relative, *Galactia*. It is notable that among these pathogens, Chupp reported *C. cruenta* (now *Pseudocercospora cruenta*) from each of the above hosts except for *Cyamopsis* and *Galactia*, and there are multiple records of this species from eighteen Fabaceae genera and also from families Lamiaceae and Solanaceae (Farr and Rossman 2015). This type of widespread geographic distribution or host generalism is also evident from collection records of *C. cf. flagellaris* and *C. cf. sigesbeckiae*, but in contrast to *C. kikuchii*, there is comparatively little information about *C. cf. flagellaris* or *C. cf. sigesbeckiae* in the literature. This is most likely because they have not been previously implicated as pathogens of economically important crops like soybean and cotton.

and Braun 2003; Kirschner 2014) and other genera in the Phytolaccaceae, including *Rivina* (Farr and Rossman 2015; Solheim and Stevens 1931) and *Petiveria* (Chupp 1954). Existing collection records show that *C. cf. flagellaris* is widespread, with reports from 24 host families distributed across seven countries (Bakhshi et al. 2015b; Groenewald et al. 2013; Kirschner 2014; Soares et al. 2015). There is no ex-type culture of *C. flagellaris* available, but the syntype is currently housed in the herbarium of the Academy of Natural Sciences in Philadelphia. Obtaining a viable culture from this material, from which DNA can be isolated and sequence data obtained, will likely not be possible. This makes the designation of an epitype culture based on an isolate collected from *P. americana* growing in or around the type locality (West Chester, PA, U.S.A.) a priority for future taxonomic studies.

Though *C. cf. flagellaris* and *C. cf. sigesbeckiae* have long been known as generalist pathogens, they have only recently been associated with soybean. Bakhshi et al. (2015b) reported *C. cf. flagellaris* from *G. max* in Iran, and later, Soares et al. (2015) reported it from Arkansas. The results from this study show that *C. cf. flagellaris* is also present in the Gulf South and in several states from the Midwest. Soares et al. (2015) did not find *C. cf. flagellaris* in tropical regions of Brazil and speculated that it might be restricted to colder climates. However, reports of *C. cf. flagellaris* from soybean grown in geographic regions that do not generally experience cold temperatures, such as Louisiana (this study), Iran (Bakhshi et al. 2015b), and also from other hosts in Ethiopia, South Africa, Fiji, Taiwan, Puerto Rico, the U.S. Virgin Islands, Florida, and Texas (Farr and Rossman 2015; Groenewald et al. 2013) suggest that *C. cf. flagellaris* is ubiquitous throughout tropical and subtropical regions as well. The many hosts from which *C. cf. flagellaris* and *C. cf. sigesbeckiae* have been isolated raises the issue of whether some of these plants, such as *Phytolacca* spp. and other “weeds” that may be present in or around soybean fields, are acting as reservoirs of inoculum. The etiology of CLB and PSS is poorly understood, and the

potential role that non-soybean hosts play in helping to spread disease has not been thoroughly investigated. *Phytoplasma* is ubiquitous, with reports from all but eight states in the United States as well as from several provinces in Canada (USDA NRCS 2016). It is commonly encountered in disturbed sites along partially shaded areas near forest borders, and is present in or around soybean fields in Louisiana (personal observation), which may help to explain how inoculum is introduced into soybean fields from year to year.

Cercospora sigesbeckiae Katsuki was described in 1949 from *Sigesbeckia glabrescens* (Asteraceae) in Japan (Chupp 1954) and is morphologically similar to species in *C. apii sensu lato* (Groenewald et al. 2013). All authenticated isolates of *C. cf. sigesbeckiae* originate from Asia, South America (Soares et al. 2015), and now the United States, where it has not been previously reported. Like *C. cf. flagellaris*, *C. cf. sigesbeckiae* has a broad host range, occurring on *G. max*, *S. pubescens*, and other hosts in six additional plant families (Farr and Rossman 2015; Groenewald et al. 2013). However, the scarcity of *C. cf. sigesbeckiae* isolates collected in this study suggests that the presence of *C. cf. sigesbeckiae* may be incidental and that *C. cf. flagellaris* is the dominant pathogen in Louisiana. Additional sampling is needed to rule out the presence of *C. kikuchii* in the United States and determine the dominant species in other soybean-growing regions.

The frequency of MAT1-1 and MAT1-2 idiomorphs in Louisiana deviated significantly from the 1:1 ratio expected for a sexually recombining population. However, we feel that these results should be interpreted cautiously when inferring a clonal *C. cf. flagellaris* population, and the results presented here should be viewed as preliminary. Because population structure within a species can impact inferences of demographic parameters, such as linkage disequilibrium, it is important to characterize gene flow among populations after careful hierarchical sampling with the use of more informative markers useful for population studies. Once populations have been delimited, the presence or absence of sexual recombination can be inferred at the population level. However, our results do indicate that the potential for sexual recombination does exist within *C. cf. flagellaris*, and further studies into the prevalence and importance of life history traits, such as sexual reproduction, are warranted.

The results of this study are relevant to systematic and taxonomic mycology as well as plant pathology and plant breeding. Management of CLB with fungicide applications has been marginal at best and disease resistance has been elusive. In a recent comprehensive study, CLB and PSS isolates from Louisiana (including many of the ones used in this study) were uniformly resistant to all the commonly used soybean fungicides, with the exception of triazoles, though a small percentage of isolates were resistant to this class of fungicide as well (Price 2013; Price et al. 2015). Integration of this information into a phylogenetic framework may help to explain the rapid development and spread of fungicide resistance and to what degree various temporal and spatial factors are involved.

Isolates of different *Cercospora* species able to cause CLB and PSS will provide breeders with theoretical and practical information as well as isolates to test in the development of durable disease resistance in new soybean lines. Otherwise, cultivars released with resistance to only a fraction of the extant pathogen diversity would likely succumb to these diseases within a relatively short time or may show geographically variable disease resistance. A diverse pathogen population may explain why previous work showed significant time and location interactions with regard to susceptibility in commercial cultivars (Cai et al. 2009). Simply put, accurate identification of a pathogen is an imperative prerequisite to developing an integrated disease management approach including genetic resistance and chemical treatment.

ACKNOWLEDGMENTS

We thank B. Bluhm, S. Sharma, J. Ridnour, S. Li and G. Cai for providing isolates used in this study and D. Nelsen, K. Weyand and P. Weyand for help with plant collections. We are also grateful to P. Muse, H. Hutchins, W. A. Vieira, D. Clement, C. Amadi, C. Robertson, B. Ward, E. C. da Silva, and T. Garcia for laboratory assistance. Finally, we thank three anonymous reviewers for helping to improve the manuscript. Some analyses were conducted using computational resources provided by High Performance Computing at Louisiana State University. Support for this research was provided in part by funding from the United Soybean Board, the Louisiana Soybean Grain and Promotion Board, and Louisiana State University Agricultural Center.

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