

# [Genetic diversity and qoi fungicide resistance](https://assignbuster.com/genetic-diversity-and-qoi-fungicide-resistance/)

Study of genetic diversity and QoI fungicide resistance in frogeye leaf spot ( Cercospora sojina ) from Tennessee

## Introduction

Frogeye leaf spot (FLS) of soybean ( Glycine max Merr.), caused by the fungal pathogen C. sojina Hara, was first identified in Japan in 1915 and South Carolina, the United States in 1924 (Lehman 1928; Phillips 1999). FLS is an important foliar disease of soybean although symptoms can appear on stems, pods, and seeds. There has been no report of the alternative host in other crops or weeds (Mian et al. 2008). Initial symptom appears as small, light brown circular spot which is later surrounded by darkish brown to reddish circle. (Dashiell and Akem 1991). As the leaves are covered with 50% lesions, leaves start to blight wither and finally falls prematurely. On the lower surface of leaves, the central spot of lesions is somewhat grayish because of conidia produced on conidiophores. Conidia are a primary and secondary source of inoculum and are produced in infected leaves, stems, and pods. Warm temperature and frequent rainfall are suitable factors for severe disease, and fully expanded leaves are more resistant with small lesions as compared to younger leaves (Phillips 1999).

The United States is the leading producer of soybean in the world. According to the food and agriculture organization (FAO), the US produced 108 million metric tons of soybeans, second only to corn in 2014 (http://faostat3. fao. org/). FLS is an important disease in most of the soybean growing countries in the world and the main factors hindering the yield includes a reduction in photosynthetic area and premature defoliation of leaves (Mian et al. 2008; Wrather et al. 2010). In the US, FLS is significantly present in Southern warm and humid regions (Mian et al. 2008; Yang et al. 2001). Now, C. sojina is also important to Northern states as the disease was reported in Iowa in 1999, Wisconsin in 2000 (Mengistu et al. 2002) and Ohio in 2006 (Cruz and Dorrance 2009). The damage caused by FLS depends on soybean cultivars and locations, and yield loss has been reported from 10% to more than 60% (Dashiell and Akem 1991; Hartwig and Edwards Jr 1990; Laviolette et al. 1970; Mian et al. 1998).

FLS is a polycyclic disease and the disease remains active throughout the growing season (Kim et al. 2013; Laviolette et al. 1970). Dispersal of conidia to some distance is favored by the wind and water splashes (Laviolette et al. 1970). Mycelium of C. sojina can overwinter and a report suggests potential survival of the pathogen in the plant debris for two years (Zhang and Bradley 2014). There are several FLS control methods including cultural practices, use of fungicides and genetic resistance. Primarily, genetic resistance is a most effective measure to control FLS. Till now, three resistant genes Rcs (Resistant to C. sojina ), have been deployed: Rcs1 (Athow and Probst 1952), Rcs2 (Athow et al. 1962) and Rcs3 (Phillips and Boerma 1982). The Rcs3 gene confers resistant against race 5 and all known races of C. sojina present in the USA. (Mengistu et al. 2012; Phillips and Boerma 1982). Similarly, crop rotation for two years has been suggested to skip viable inoculum and prevent disease severity in the field (Grau et al. 2004; Zhang and Bradley 2014). Further, use of pathogen-free seeds and necessary application of fungicides before flowering to early pod stage have been practiced to decrease disease severity (Grau et al. 2004). Meanwhile, because of change in the pathogen, it has been proven that resistant gene can confer resistance for a certain period and there can be selection against QoI fungicides too (Athow and Probst 1952; Athow et al. 1962; Zeng et al. 2015). There has already been a report of field isolates resistant to QoI fungicides in Tennessee (Zhang et al. 2012). Control measures like use of fungicides and planting of resistant cultivars force pathogens to select against selection pressure.

Studies of C. sojina using several approaches indicate diversity among isolates. Because of the lack of universally accepted soybean differentials, it’s hard to characterize and compare C. sojina isolates. Grau et. al. (2004) have reported 12 races of C . sojina in the US, 22 races in Brazil and 14 races in China. A new set of 12 soybean differentials and 11 races have been proposed based on the reaction of isolates collected from the USA, Brazil, and China (Mian et al. 2008). However, the reaction of 50 isolates from Ohio on the same 12 soybean differentials produced 20 different races (Cruz and Dorrance 2009). There has been a handful of research to characterize C. sojina based on molecular markers. One study includes AFLP based assessment of 62 isolates from Brazil, China, Nigeria and the United States, which showed a significant amount of genetic diversity among isolates, although genotypes did not cluster based on origin. (Bradley et al. 2012). Recently, a study of 132 isolates from Arkansas with simple sequence repeat (SSR) has shown the chances of sexual reproduction and high genetic diversity in C. sojina (Kim et al. 2013).

The main objectives of this study were to access: genetic diversity by developing and using novel SNP markers and distribution of QoI resistant and sensitive isolates from Jackson and Milan, TN.

## Sample collection, Single-lesion Isolation, and DNA extraction

In 2015, soybean leaves exhibiting typical symptoms of infection with FLS were collected from research plots at two locations in Tennessee (Milan and Jackson). In total, 437 isolates, 203 from Jackson and 234 from Milan, were collected from eight fungicides treated and non-treated Maturity group III soybean cultivars (Table 1). Cultivars were planted in 4 rows (30-inch row spacing), 30 ft long plots in a randomized complete block design with four replications. Plots were split, two rows were not treated, and two rows were treated at R3 growth stage (beginning pod) with Quadris Top SB at 8 fl oz/a (Azoxystrobin and Difenoconazole, Syngenta Corp., Basel, Switzerland). A single isolate of C. sojina was obtained from a single lesion from each leaf. Sporulation was induced by incubating leaves in a plastic bag with moist towels at room temperature. Spores were harvested with a flame-sterilized needle using a dissecting microscope and 8-10 spores transferred to RA-V8 agar media (rifampicin 25 ppm, ampicillin 100 ppm, 160 mL unfiltered V8 juice, 3 gm calcium carbonate and 840 mL water). Observations were made daily and contaminated sectors removed. After seven days, single-lesion isolates of C. sojina were transferred to a new V8 agar media. In addition, a set of 40 isolates from 10 different states, collected before 2015, were included in this study (Table 2).

Table 1. Soybean cultivars and number of Cercospora sojina isolates recovered from treated and non-treated cultivars.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cultivar ID  | Cultivars  | Jackson  | Milan  | Total  |
| Treated  | Non-treated  | Treated  | Non-treated  |
| C1  | VAR Armor 37-R33 RR2  | 17  | 11  | 21  | 4  | 53  |
| C2  | VAR Asgrow AG3832 GENRR2Y  | 7  | 15  | 20  | 14  | 56  |
| C3  | VAR Beck’s 393R4  | 0  | 0  | 0  | 3  | 3  |
| C4  | VAR Croplan R2C 3984  | 19  | 13  | 11  | 14  | 57  |
| C5  | VAR Mycogen 5N393R2 RR2 g  | 12  | 20  | 17  | 28  | 77  |
| C6  | VAR Terral REV 39A35  | 10  | 15  | 13  | 16  | 54  |
| C7  | VAR USG 73P93R  | 22  | 6  | 13  | 21  | 62  |
| C8  | VAR Warren Seed 3780 R2Y It  | 14  | 22  | 13  | 26  | 75  |

Table 2. Number of Cercospora sojina isolates collected from Jackson (JTN) and Milan (MTN), Tennessee in 2015 and historical isolates from various states in previous years.

|  |  |  |
| --- | --- | --- |
| Location  | No. of Samples  | Year  |
| JTN  | 203  | 2015  |
| MTN  | 234  | 2015  |
| AL  | 5  | 2006  |
| AR  | 5  | 2006  |
| FL  | 1  | 2006  |
| GA  | 4  | 2006  |
| IA  | 1  | 2006  |
| IL  | 2  | 2006/09  |
| LA  | 1  | 2006  |
| MS  | 6  | 2006  |
| SC  | 2  | 2006/2009  |
| TN  | 12  | 2007  |
| WI  | 1  | 2006  |

Note: JTN (Jackson) and MTN (Milan) collection in 2015 in Tennessee. TN is a historical collection.

For DNA extraction, the single-lesion isolates were grown in 24-well deep well plates (Fisher Scientific) with 1 mL RA-V8 liquid broth (same as above, minus the agar) per well. DNA was extracted as described by Lamour and Finley (2006). Briefly, this includes harvesting mycelium from the broth cultures into a 96-well 2 mL deep well plate pre-loaded with 3-5 sterilized 3 mm glass beads. The plates are freeze dried and the dried mycelium powdered using a Mixer-Mill bead beating device (Qiagen). The powdered mycelium was then lysed and a standard glass fiber spin-column DNA extraction completed. The resulting genomic DNA was visualized on a 1% gel and quantified using a Qubit device.

## SNP marker discovery and targeted-sequencing based genotyping

Whole genome sequencing was accomplished for three FLS isolates from a historical collection originally compiled by Dr. Dan Philips, UGA: FLS11 (CS10117) recovered from Milan, Tennessee in 2010, FLS19 (TN10) from the Georgia Experiment Station, and FLS21 (TN85) which was recovered from Mississippi. Genomic DNA was extracted from freeze-dried and powdered mycelium using a standard phenol-chloroform approach and the resulting DNA was submitted to the Beijing Genomics Institute in China for 2×100 paired-end sequencing on an Illumina HiSeq2000 device. De novo assembly, read mapping and SNP discovery was accomplished with CLC Genomics Workbench 7 (Qiagen). As there was no public reference genome available at the time, FLS21 was de novo assembled using the default settings in CLC and the resulting contigs used as a reference genome. All open reading frames (ORFs) longer than 300 amino acids were predicted using CLC and annotated onto the FLS21 contigs. The raw reads from FLS11 and FLS19 were then mapped to the draft reference (separately), and putative single nucleotide variants (SNVs) identified at sites with at least 20X coverage and an alternate allele frequency greater than 90%.

A subset of the SNVs was chosen from the largest contigs for further genotyping using a targeted sequencing approach. Custom Perl scripts were used to extract the flanking sequences for the panel of SNPs and primers were designed using BatchPrimer3 v1. 0 (http://probes. pw. usda. gov/batchprimer3/) to amplify targets between 80 and 120bp in length. Primers for 50 SNPs including mitochondrial QoI resistant locus are summarized in Table 3. Primer sequences and genomic DNA were sent to Floodlight Genomics (Knoxville, TN) for processing as part of a non-profit Educational and Research Outreach Program (EROP) that provides targeted-sequencing services at cost for academic researchers. Floodlight Genomics uses an optimized Hi-Plex approach to amplify targets in multiplex PCR reactions and then sequences the resulting sample-specific amplicons on either an Illumina or Ion NGS device. Resulting sample-specific sequences were mapped to the reference contigs and genotypes assigned for loci with at least 6X coverage.

## QoI resistant locus genotyping

A single nucleotide polymorphism (G/C) in the Cytochrome b gene of the C. sojina mitochondrial genome has been shown to confer resistance to QoI fungicides. A custom TaqMan SNP genotyping assay will be designed using the online design tools from Applied Biosystems (Thermo Scientific) and include the forward primer GGGTTATGTTTTACCTTACGGACAAATG and reverse primer GTCCTACTCATGGTATTGCACTCA and two probes to discriminate resistant and sensitive isolates: ACTGTGGCA G CTCATAA with VIC for the “ C” resistance allele and ACTGTGGCA C CTCATAA with FAM for the “ G” sensitive allele (Zeng et al. 2015). Quantitative PCR (qPCR) will be accomplished based on manufacturer instruction using the QuantStudio 6 Flex Real-time PCR System (Thermo Fisher Scientific Inc.).

## Mating types determination

A previously described multiplex PCR assay will be used to assign mating type (MAT1-1-1 or MAT1-2) to a subset of the isolates that had unique multi-locus SNP genotypes (Kim et al. 2013). The MAT1-1-1 locus will be amplified with CsMat1f (5′ TGAGGACATGGCCACCCAAATA) and CsMat1r (5′ AAGAGCCCTGTCAAGTGTCAGT) and the Mat1-2 locus will be amplified with CsMat2f (5′ TGTTGTAGAGCTCGTTGTTCGCA) and CsMat2r (5′ TCAGACCTTATGAGCTTGAAAGTGCT) primers (Kim et al. 2013). The assay will be included with the ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′ TCCTCCGCTTATTGATATGC ) primers as an internal control to amplify the internal transcribed spacer (ITS) region (White et al. 1990). The resulting PCR products will be visualized under UV light on 2% agarose gel stained with GelRed (Phenix Research Products) and scored based on fragment size of MAT1-1-1 (405 bp) and Mat1-2 (358 bp).

## Data Analysis

SNP loci for each sample will be combined to form a multi-locus SNP genotypes and samples with identical genotype (clonal lineages) will be clone corrected. To assess population structure among the two locations (and in relation to the historical isolates), Bayesian clustering will be accomplished using Structure 2. 3. 4 (Pritchard et al. 2000). Structure Harvester (Earl 2012) will be used to find the most probable value of K from the results obtained from Structure analysis. Principle coordinate analysis, AMOVA, Nei pairwise genetic distance, Nei pairwise genetic identity and genetic indices will be analyzed with GENALEX (Peakall and Smouse 2006). Phylogenetic clustering of the unique genotypes will be accomplished using Mega 6. 06 (Tamura et al. 2013). Minimum spanning networks (Bandelt et al. 1999) will be constructed with PopART (http://popart. otago. ac. nz/).

## Expected Results

Novel SNP markers will be developed and assayed in C. sojina isolates. Population study will help to determine if the isolates from two locations are sub-grouped. The genetic study will also accesses genetic diversity present within and among populations.

Molecular identification of mutated cytochrome b site will help to determine the distribution of resistant isolates and contribute to compare resistant isolates in fields between two different time periods. Study of two different mating types in population will help to predict sexual reproduction.

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