Genetic Structure of *Rhizoctonia solani* AG-2-2IIIB from Soybean in Illinois, Ohio, and Ontario

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ABSTRACT

Rhizoctonia solani AG-2-2IIIB is an important seedling pathogen of soybean in North America and other soybean-growing regions around the world. There is no information regarding the population genetics of field populations of *R. solani* associated with soybean seedling disease. More specifically, information regarding genetic diversity, the mode of reproduction, and the evolutionary factors that shape different *R. solani* populations separated in time and space are lacking. We exploited genotyping by sequencing as a tool to assess the genetic structure of *R. solani* AG-2-2IIIB populations from Illinois, Ohio, and Ontario and investigate the reproductive mode of this subgroup. Our results revealed differences in genotypic diversity among three populations, with the Ontario population having greatest

diversity. An overrepresentation of multilocus genotypes (MLGs) and a rejection of the null hypothesis of random mating in all three populations suggested clonality within each population. However, phylogenetic analysis revealed long terminal multifurcating branches for most members of the Ontario population, suggesting a mixed reproductive mode for this population. Analysis of molecular variance revealed low levels of population differentiation, and sharing of similar MLGs among populations highlights the role of genotype flow as an evolutionary force shaping population structure of this subgroup.

Keywords: mycology, population biology

The soilborne necrotrophic fungus *Rhizoctonia solani* (synonym *Thanatephorus cucumeris*) is a species complex comprising a genetically diverse group of pathogens that have been associated with diseases of economically important crops worldwide (Sneh et al. 1991). Fourteen genetically isolated groups, referred to as anastomosis groups (AGs), have been differentiated, many of which have been further divided into subgroups based on various phenotypic, biochemical, and molecular markers (Carling et al. 1999, 2002a, b; Sneh et al. 1991). *R. solani* anastomosis group 2, subgroup 2IIIB (AG-2-2IIIB) is a major seedling pathogen of soybean in North America (Dorrance et al. 2003; Liu and Sinclair 1991; Muyolo et al. 1993; Nelson et al. 1996; Zhao et al. 2005) and other parts of the world where soybean is grown (Fenille et al. 2002). Disease symptoms, which include seed decay and preemergence damping off, are favored by cool and wet conditions that occur after planting, and they are often prevalent in fields with a history of disease. On emerged seedlings, hypocotyl rot, with characteristic sunken reddish brown lesions, is commonly observed, and it is most

often accompanied by root rot, which compromises the plant root system and may result in plant death.

Knowledge of pathology and host range of *R. solani* AG-2-2IIIB has accumulated over the years; however, less is known with respect to the ecology and population genetic structure at the field and regional scales. *R. solani* AG-2-2IIIB possesses saprophytic ability and can produce long-lived “nutrient-independent propagules” called sclerotia (Hoitink et al. 1991), which allow survival for long periods in the absence of a suitable host plant. Reproduction is mostly asexual, and vegetative structures, mycelia and sclerotia, are associated with infection and dispersal. Although sexual reproduction in some *R. solani* AGs is well documented (Ceresini et al. 2002; Lee and Rush 1983), field studies documenting basidiospore production of AG-2-2IIIB on soybean are lacking; therefore, the role that sexual reproduction plays in the disease cycle and creating and maintaining diversity among populations of this subgroup on this host is unknown.

Knowledge of the amount and distribution of genetic diversity within and among populations of different *R. solani* AGs is of practical significance, especially in making predictions about evolutionary response to different disease management practices (McDonald and McDermott 1993). Moreover, an understanding of population genetic structure of different AGs or subgroups can provide important clues to mating systems and reproductive modes that have shaped populations over time. Previous population genetic studies of *R. solani* have been hampered mostly by difficulties in inducing the sexual stages of field isolates in vitro and difficulty in distinguishing, in the absence of clamp connections, multinucleate homokaryons from heterokaryons (Cubeta and Vilgalys 1997). Nonetheless, the population structures of a number of AGs and subgroups, especially those associated with economically important crops, have been studied using different molecular markers. Using restriction fragment-length polymorphic (RFLP) markers, Rosewich et al. (1999) provided evidence for sexual reproduction

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among populations of AG-1-IA recovered from six major rice-growing counties in Texas. In another study using simple sequence repeat (SSR) markers, Bernardes de Assis et al. (2008) found that the population structure of AG-1-IA isolates from soybean and rice in Louisiana was characterized by both sexual and asexual reproduction. A mixed reproductive system has been reported for AG-3 (Ceresini et al. 2002; Ferrucho et al. 2013), whereas populations of AG-8 are assumed to be predominantly clonal (MacNish et al. 1993). In addition, amplified fragment-length polymorphism (AFLP) analysis of field isolates of AG-2-2IV and their single basidiospore progeny have provided evidence for both homothallic and heterothallic mating behaviors in this subgroup. Mating system and reproductive modes of AG-2-2IIIB are unknown, although Cubeta and Vilgalys (1997) suggested heterothallism and a mixed reproductive mode.

Diversity quantification and assessment in plant pathogenic fungi have, over the years, been accomplished using a variety of phenotypic and molecular markers, including vegetative compatibility groups, fungicide resistance, mating type, allozymes, RFLPs, random amplified polymorphic DNA, and AFLPs (Brown 1996); however, microsatellites (SSRs) and single-nucleotide polymorphisms (SNPs) are preferred markers (Milgroom 2015). For population genetic studies, an ideal marker should be polymorphic among populations under study, be locus specific, be unlinked (linkage equilibrium), possess minimal homoplasy, allow scoring of heterozygotes (codominant), be repeatable with unambiguous scoring, and not be associated with a phenotype under selection (Milgroom 2015). Although both SSR and SNP markers have many of these characteristics, use of SNPs for the study of genetic diversity among fungal populations is receiving more attention because of new approaches for reduced representation genome sequencing using next generation sequencing technologies that enable the simultaneous discovery and genotyping of thousands of markers in multiple individuals. Genotyping by sequencing (GBS) (Elshire et al. 2011) represents one such approach for reducing genome representation to allow simultaneous discovery and genotyping of SNPs, and its application in population genetic studies of fungi is well documented (Milgroom et al. 2014; Wilson et al. 2015).

Given the importance of AG-2-2IIIB as a pathogen of soybean and the lack of information about mating systems in this subgroup as well as the lack of information on the diversity among field populations, we sought to exploit GBS as a tool to examine the population structure of AG-2-2IIIB isolates to enable us to make inferences regarding the role of asexual and sexual reproduction in creating diversity and provide insight into the evolutionary factors that shape the population structures of different field populations. Using populations of AG-2-2IIIB from Illinois, Ohio, and Ontario, we conducted analyses to answer the following questions. (i) What is the genetic structure of the population of AG-2-2IIIB recovered from soybean seedlings across different geographical locations? Is the observed genetic structure consistent with that of a pathogen that reproduces sexually or asexually? (ii) What is the reproductive mode of the AG-2-2IIIB? Is it sexual or asexual, or is there evidence for a mixed reproductive system? Considering that members of this subgroup are considered to be largely clonal, we additionally sought evidence of recombination in addition to detecting departures from random mating. (iii) Are the populations of AG-2-2IIIB from Illinois, Ohio, and Ontario genetically differentiated, or do the isolates represent a large interbreeding population?

MATERIALS AND METHODS

Fungal isolates. Fifty-nine *R. solani* AG-2-2IIIB isolates used in the study were recovered from symptomatic soybean seedlings sampled across fields in Illinois ($n = 35$), Ohio ($n = 7$), and Ontario, Canada ($n = 17$) (Table 1). The Illinois isolates were collected between 1991 and 2013 (Ajayi-Oyetunde and Bradley 2017;

Liu and Sinclair 1991). The single isolate (65L-2) from Illinois that was recovered in 1991 represents the gold standard specimen for rhizoctonia seedling disease research in soybean (Ajayi-Oyetunde and Bradley 2017; Bradley et al. 2001, 2002), and therefore, it was included as a reference isolate. The Ohio isolates were collected between 1991 and 1999 (Dorrance et al. 2003), and the isolates from Ontario were collected in 2013. Although the number of isolates from Ohio was too small to constitute a population, these older isolates were included, similar to isolate 65L-2 from Illinois, as checks to provide a baseline for assessing genotypic diversity and reproductive mode. The Illinois and Ontario isolates were determined previously to be members of AG-2-2IIIB using conventional and molecular techniques (Ajayi-Oyetunde and Bradley

TABLE 1. Origin, year of isolation, and multilocus genotypes (MLGs) of *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB isolates recovered from soybean seedlings in Illinois, Ohio, and Ontario

Isolate identification	Origin	Year	Genotype
12Rs41	Williamson Co., Illinois	2012	MLG.12
211_1b	Champaign Co., Illinois	2012	MLG.7
42210_b	Unknown, Illinois	2013	MLG.10
42210_c	Unknown, Illinois	2013	MLG.10
42304_b	Unknown, Illinois	2013	MLG.10
42304_c	Unknown, Illinois	2013	MLG.10
42304_g	Unknown, Illinois	2013	MLG.10
42304_h	Unknown, Illinois	2013	MLG.10
65L-2	Champaign Co., Illinois	1991	MLG.3
DK_10	DeKalb Co., Illinois	2013	MLG.9
DK_11	DeKalb Co., Illinois	2013	MLG.17
DK_13	DeKalb Co., Illinois	2013	MLG.17
DK_14	DeKalb Co., Illinois	2013	MLG.17
DK_15a	DeKalb Co., Illinois	2013	MLG.17
DK_15B	DeKalb Co., Illinois	2013	MLG.17
DK_16	DeKalb Co., Illinois	2013	MLG.17
DK_18	DeKalb Co., Illinois	2013	MLG.17
DK_19	DeKalb Co., Illinois	2013	MLG.10
DK_3a	DeKalb Co., Illinois	2013	MLG.17
DK_3b	DeKalb Co., Illinois	2013	MLG.18
DK_4a	DeKalb Co., Illinois	2013	MLG.17
DK_4b	DeKalb Co., Illinois	2013	MLG.17
DK_6a	DeKalb Co., Illinois	2013	MLG.17
DK_6b	DeKalb Co., Illinois	2013	MLG.17
DK_7	DeKalb Co., Illinois	2013	MLG.17
DK_8	DeKalb Co., Illinois	2013	MLG.17
ER_15	Whiteside Co., Illinois	2013	MLG.17
ER_19a	Whiteside Co., Illinois	2013	MLG.8
ER_19b	Whiteside Co., Illinois	2013	MLG.8
ER_2	Whiteside Co., Illinois	2013	MLG.8
ER_4	Whiteside Co., Illinois	2013	MLG.8
K_ILSO2_3_25a	Warren Co., Illinois	2013	MLG.8
K_ILSO2_3_25b	Warren Co., Illinois	2013	MLG.2
K_ILSO2_3_25c	Warren Co., Illinois	2013	MLG.3
Rs1039	Champaign Co., Illinois	2000	MLG.3
91_1	Unknown, Ohio	1991	MLG.13
99_12	Pickaway Co., Ohio	1999	MLG.3
99_13	Morrow Co., Ohio	1999	MLG.3
99_14	Unknown, Ohio	1999	MLG.17
99_15	Wood Co., Ohio	1999	MLG.17
99_18	Unknown, Ohio	1999	MLG.16
PL_1	Unknown, Ohio	Unknown	MLG.10
WONS 2013-12-3	Rodney, Ontario	2013	MLG.11
WONS 2013-8-2	Merlin, Ontario	2013	MLG.15
WONS 2013-8-6	Merlin, Ontario	2013	MLG.6
PDONS 2013-8-4	Merlin, Ontario	2013	MLG.1
PDONS 2013-8-5	Merlin, Ontario	2013	MLG.5
PDONS 2013-8-6	Merlin, Ontario	2013	MLG.3
PDONS 2013-8-3	Merlin, Ontario	2013	MLG.1
PDONS 2013-8-7	Merlin, Ontario	2013	MLG.15
WONS 2013-8-8	Merlin, Ontario	2013	MLG.15
WONS 2013-8-7	Merlin, Ontario	2013	MLG.17
WONS 2013-8-1	Merlin, Ontario	2013	MLG.14
WONS 2013-8-5	Merlin, Ontario	2013	MLG.4
PDONS 2013-8-1	Merlin, Ontario	2013	MLG.14
PDONS 2013-12-6	Rodney, Ontario	2013	MLG.19
WONS 2013-8-4	Merlin, Ontario	2013	MLG.1

2017). Although isolates from Ohio were reported to be AG-2-2IIIB, they were further characterized by PCR amplification with AG-2-2IIIB-specific primers (Carling et al. 2002a, b) and PCR-RFLP of the internal transcribed spacer regions of the ribosomal genes as described by Guillemaut et al. (2003). Isolates were initially maintained on potato dextrose agar (PDA) amended with rifampicin at 25 mg/liter. After 5 days of growth, isolates were prepared for long-term storage at -80°C by transferring five 5-mm plugs from PDA plates to 1.5-ml microcentrifuge tubes containing 850 μl of 15% glycerol.

DNA extraction, SNP library construction, and genotyping. For genomic DNA extraction, each isolate was grown at room temperature ($\sim 25^{\circ}\text{C}$) in 100 ml of potato dextrose broth (Difco Laboratories) for 14 days without shaking. Mycelia were harvested, and DNA was extracted using FastDNA spin kits (MP Biomedicals). DNA of each isolate was diluted to 40 ng/ μl and confirmed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). SNP libraries were developed for each isolate with slight modification of protocols described previously (Poland et al. 2012). A pilot restriction digest with four enzyme combinations (*Pst*I-*Hin*PII, *Pst*I-*Bfa*I, *Hind*III-*Hin*PII, and *Hind*III-*Bfa*I) was initially conducted on eight random samples to identify the best restriction enzyme combination for *R. solani*. Enzymes *Pst*I and *Hind*III are rare cutters, whereas *Hin*PII and *Bfa*I are common cutters. The *Hind*III-*Hin*PII combination was selected based on the absence of adapter dimers. For each of the 59 samples, the restriction-ligation reaction was performed in a 25- μl reaction volume containing 5 μl (200 ng) of genomic DNA, 2.5 μl of 10 $\times$ NEB CutSmart buffer, 2.5 μl of 10 mM dATP, 0.1 μl (2 U) of *Hind*III, 0.2 μl of *Hin*PII, 0.1 μl of concentrated T4 DNA ligase (40 U), 0.5 μl of 10 μM Adapter2, and 14.1 μl of molecular-grade sterile water. Restriction digest was run at 37°C for 2 h followed by heat inactivation at 80°C for 15 min, whereas ligation reaction was performed at 25°C for 2 h followed by heat inactivation at 65°C for 20 min.

DNA from barcoded libraries was pooled and cleaned using a 1:1 ratio of AmpureXP Beads (Beckman Coulter) to DNA, washed twice in 80% ethanol, and suspended in 15 μl of 10 mM Tris elution buffer. PCR amplification of the purified pooled DNA was conducted in a 50- μl reaction composed of 3 μl of DNA, 2 μl of Illumina primer mix (forward and reverse), 25 μl of Phusion High Fidelity PCR Master Mix (New England Biolabs), and 20 μl of water. Cycle parameters for PCR amplification were 98°C for 30 s; 18 cycles of 98°C for 10 s, 68°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. Amplification products were subjected to a second cleanup as described above followed by quantification of DNA fragment size and concentration using an Agilent 2100 Bioanalyzer and Agilent DNA 7500 kit (Agilent Technologies Inc.). Two separate libraries simultaneously constructed were diluted to 10 nM and pooled before submission for single-end 100-bp sequencing using the Illumina HiSeq2000 at the W. M. Keck Center (University of Illinois, Urbana, IL). The Illumina sequence data from this study have been submitted as BioProject identification PRJNA544472 to the NCBI Sequence Read Archive.

Raw reads were obtained as Fastq files, with sequence reads matched to sample identification number using the DNA barcode sequence, and they were subsequently trimmed from sequence reads. For alignments and identification of SNPs, the consensus genome sequence of *R. solani* AG-3 strain Rhs1AP (Cubeta et al. 2014) was downloaded from the NCBI (<https://www.ncbi.nlm.nih.gov/>) for use as a reference genome. However, because no reads aligned to this genome, likely because of insufficient sequence similarity of the AG-2-2IIIB to the AG-3 genome, reads were assembled, and SNPs were called using the Universal Network Enabled Analysis Kit pipeline (Lu et al. 2013). Minimum minor allele frequency was 5%, and minimum call rate per each locus was set to 0.5 (i.e., $\leq 50\%$ missing data per locus), resulting in a total of 4,733 SNPs. Subsequently, monomorphic loci and loci with $>10\%$

missing data were excluded as were two isolates with $>10\%$ missing data. Remaining missing data were imputed to allele frequency. A total of 57 genotypes with 2,019 SNP loci were used for final data analysis, and data were formatted in Excel following the specified format for analyzing codominant genotypic data in GenAlEx 6.5 (Peakall and Smouse 2012).

Multilocus genotypes, diversity, and population differentiation. A multilocus genotype (MLG) is a unique combination of alleles from multiple independent loci (Milgroom 1996). To eliminate small differences between MLGs, pairwise genetic distance between genotypes was calculated using Nei's genetic distance (H_{exp}), and the unweighted pair group method with arithmetic mean (UPGMA; average neighbor) was selected as clustering algorithm to determine which genotypes cluster together and can be considered clones. This procedure was performed in R using the function *mlg.filter* from the R package *poppr* version 2.8.1 (Kamvar et al. 2014). This identified $H_{exp} \geq 0.030$ was the optimal threshold to determine if two genotypes were unique, which collapsed the set of MLGs from 57 to 19.

Because *R. solani* AG-2-2-IIIB is considered to be functionally asexual and because populations are assumed to exhibit a clonal structure that is maintained from one generation to the next via asexual reproduction, we began diversity assessment by determining the genotypic diversity within each population. Genotypic diversity is a measure of genotypic richness and evenness. Genotypic richness is the number of genotypes in a population. Estimates of the number of MLG increase for populations with larger sample sizes, making comparison of genotypic richness between populations with unequal samples sizes biased. Therefore, comparisons were made using the expected number of multilocus genotype (eMLG), which uses rarefaction to calculate number of MLGs expected at a sample size equivalent to the smallest sample size (Grünwald et al. 2003).

Genotypic evenness is a measure of the relative abundance of each genotype. The distribution of genotypes, otherwise referred to as genotypic evenness E_5 , was calculated as the ratio of the number of abundant genotypes to the number of the rarer genotypes (Grünwald et al. 2003). Genotypic diversity estimates were calculated using *poppr*.

Using the nonclone-censored data containing 19 MLGs, indices of genotypic diversity for each population, including Stoddart and Taylor's diversity index G_0 (expressed as $G_0 = 1/\sum p_i^2$, where p_i is the frequency of the i th genotype), Simpson's diversity index λ (expressed as $\lambda = 1/\sum p_i^2$, where $\sum p_i^2$ is the sum of the squared genotype frequencies), and Shannon-Weiner index (H'), were determined using *poppr*. To compare diversity statistics among populations, confidence intervals for G_0 , λ , and E_5 were obtained using the *diversity_ci* function in *poppr*.

Minimum spanning networks were constructed to assess the genetic relatedness among MLGs for the Illinois population and the three geographical regions combined. MLGs were generated using the *imsmn* function in *poppr*, with genetic distance between MLGs determined using Nei's distance. The clonal fraction, which is a measure of the proportion of genotypes in a population originating from asexual reproduction, was calculated as $1 - [(\text{number of different genotypes}/(\text{total number of isolates}))]$ (Zhan et al. 2003) and estimated for each population. For downstream analyses, isolates having the same MLG were considered clones, and data were clone censored (using the *clonecorrect* function in *poppr*) so that isolates with the same MLG were represented only once.

An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was conducted to determine if the populations were genetically differentiated. First, we calculated Φ_{PT} , an analog of F_{ST} that does not take into account variation within individual isolates, to determine if most of the genetic variation observed among the isolates was owing to genotypic differences within or among populations. Second, we determined the overall population

genetic differentiation, F_{ST} , which partitions the genetic diversity observed among all of the individuals to diversity among the populations (F_{ST}), among individuals (F_{IS}), and within individuals (F_{IT}). For this analysis, the null and alternative hypotheses were

$$H_0 : F_{ST} = 0 \text{ (i.e., no genetic differentiation among populations)}$$
$$H_1 : F_{ST} > 0 \text{ (i.e., populations are subdivided)}$$

Statistical significance of F_{ST} was based on 9,999 permutations, and a significant genetic differentiation among populations was declared when $P \leq 0.05$. AMOVA and significance testing were conducted in GenAlEx 6.5. AMOVA was conducted on both clone-censored and nonclone-censored data. Because of sample size reduction after clone censoring, the Ohio population was not included in this analysis.

Reproductive mode and population structure. To address the question about the reproductive biology of *R. solani* AG-2-2IIIB, three approaches were used to ascertain whether there was evidence that the populations were the product of primarily asexual or sexual reproduction. First, we conducted tests to determine associations among SNP loci as an indication of whether populations were in linkage disequilibrium (LD). The index of association I_A , which is based on variance in the number of alleles k at which two pairs of MLGs differ, is a useful metric for testing multilocus LD (Brown et al. 1980). Under conditions of random mating, the observed variance s_k^2 should approach the expected variance σ_k^2 , producing an I_A of 0 (where $I_A = \frac{s_k^2}{\sigma_k^2} - 1$) (Milgroom 1996). However, the value of I_A increases with the number of loci examined, making comparison among populations difficult; hence, a standardized index of association $\bar{r}_d$, which is independent on the number of loci and can, as a result, permit comparison across datasets, is often preferred over I_A for the estimation of the extent of LD. Estimates of I_A and $\bar{r}_d$ were obtained and used to test the null hypothesis that the loci are unlinked (i.e., are randomly mating). Significance testing of estimates of I_A and $\bar{r}_d$ were made using 1,000 permutations, wherein P values of ≥ 0.05 indicated no LD. Estimation of I_A and $\bar{r}_d$ for both uncensored and clone-censored data and tests for significance were performed using the *poppr* package in R. LD was not evaluated for the Ohio population because of small sample size after clone censoring.

Second, in the next approach, we used branching patterns (topology) and lengths of phylogenetic trees as indicators of sexually recombined populations. This was based on the assumption that sexual recombination results in identical alleles with different evolutionary histories, evidenced by incongruent phylogenetic trees or trees that lack resolution (Burt et al. 1996; Milgroom 1996). Following this expectation, sexual populations should produce multifurcating branches with long tree lengths, whereas clonal populations should produce bifurcating trees with short tree lengths. Bootstrapped dendrograms were calculated using the function *aboot* of the *poppr* package, and phylogenetic trees were constructed using the function *plot.phylo* in the R package *ape*. Bootstrap analysis was performed using 1,000 bootstrap replicates, and Nei's genetic distance was used for calculating genetic distances between isolates.

Third, this approach determined the inbreeding coefficient, F_{IS} , for each population to test the hypothesis of recombination between related individuals as a possible cause of deviation from the expectation of random mating (sexual reproduction). Mating systems in organisms that reproduce sexually can vary from strict inbreeding (homothallic) to predominantly outcrossing (heterothallic). Populations that undergo inbreeding or mating among closely related individuals usually are typically characterized by a deficit of heterozygous loci compared with populations that are randomly mating because of sharing of alleles among individuals

within the population. The inbreeding coefficient is a standard measure of identity and yields the probability that alleles chosen at random from a population are identical by descent. This value ranges from 0 to 100%, where a value of 0 indicates that there is no inbreeding. The inbreeding coefficient was calculated in ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010), and P values ≤ 0.05 indicated a significant deviation from random mating.

TABLE 2. Genotypic diversity and linkage disequilibrium in *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB populations from Illinois, Ohio, and Ontario

Indices/metrics	Ontario	Illinois	Ohio
<i>N</i>	15	35	7
Genotypic diversity			
Multilocus genotypes ~ (richness)	10	9	5
Expected number of multilocus genotypes (expected richness)	7.53	4.84	5.0
Shannon's diversity (<i>H'</i>)	2.18	1.68	1.55
Stoddard and Taylor's genotypic diversity (<i>G</i>)	7.76	3.91	4.45
Simpson's diversity (λ)	0.87	0.74	0.78
<i>E</i> ₅ (evenness)	0.87	0.67	0.93
Clonal fraction: 1 – (multilocus genotype/ <i>N</i>)	0.33	0.74	0.29
Linkage disequilibrium			
<i>I</i> _A ^a	198.10 ^b	401.84 ^b	280.03 ^b
$\bar{r}_d$ ^a	0.117 ^b	0.233 ^b	0.183 ^b
<i>I</i> _A ^c	157.06 ^b	151.88 ^b	– ^d
$\bar{r}_d$ ^c	0.094 ^b	0.088 ^b	–

^a Estimates for uncensored data.

^b Significance at $P < 0.0001$.

^c Estimates for clone-censored data.

^d Dashes indicate no estimate.

Fig. 1. Minimum spanning network showing the multilocus genotypes (MLGs) observed in the Illinois, Ohio, and Ontario populations of *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB and the relationship among the MLGs. Each node/circle represents a different MLG, and the number inside each node represents the frequency of that MLG. Node color represents population membership. Connecting nodes are edges, wherein edge thickness and color are proportional to Nei's genetic distance indicated in the scale bar.

Population structure or admixture has been recognized as a potential cause of LD. An admixed population can arise from a large migration event or when two genetically differentiated subpopulations are treated as a single undifferentiated population. Therefore, an assessment of population structure is necessary to help validate conclusions of lack of sexual recombination or no LD. The genetic structures of the combined Illinois, Ohio, and Ontario populations were assessed using both the uncensored data and the clone-censored data. Population structure was examined by conducting a discriminant analysis of principal component (DAPC) to detect admixed genotypes or individuals. DAPC is a multivariate analysis method that combines the strengths of principal component analysis (PCA) and discriminant analysis to investigate the genetic structure of populations by maximizing the between-population variability and minimizing the within-population variability (Jombart et al. 2010). Similar to PCA, DAPC does not rely on the assumptions of random mating (or

Hardy–Weinberg equilibrium) or LD, and it can be applied to both sexually and asexually reproducing populations. For DAPC analysis, a cross-validation analysis was conducted to determine the appropriate number of principal components to retain (based on mean squared error) that would explain most of the variability among the defined population. Cross-validation and DAPC analysis were conducted using the adegenet package in R.

RESULTS

Genotypic diversity and population differentiation. A total of 19 MLGs were identified among the 57 isolates evaluated (Illinois: 35; Ohio: 7; and Ontario: 15). The number of MLGs varied across populations, with the Ontario population having the highest number (10) followed by Illinois (9) and Ohio populations (5) (Table 2). Two MLGs were present in all three populations, whereas one MLG was present in both the Illinois and Ohio populations (Fig. 1). Genotypic richness and overall genotypic diversity were highest for the Ontario population, whereas evenness was highest for the Ohio populations (Table 2). The Illinois and Ohio populations were comparable in terms of richness and genotypic diversity; however, the former had a lower genotypic evenness. Percentage clonality was low for the Ontario and Ohio populations but high for the Illinois population.

An examination of distribution of MLGs in Illinois (Fig. 2) revealed that nine MLGs were shared across five of six counties where isolates were recovered. The single isolate representing Williamson County (IL-WI) had a unique MLG. DeKalb County (IL-DK) had the highest number of MLGs: four MLGs from 17 isolates. One of these MLG was also identified in Whiteside County (IL-UN), and another was shared with isolates from an unidentified county (IL-UN). Warren County (IL-WA) isolates had three MLGs, two of which were shared with Champaign County (IL-CH) and Whiteside County (IL-WS). Two MLGs were detected among isolates from Champaign County (IL-CH). The MLG of the reference isolate 65L-2 (MLG.3) that was initially recovered in Champaign County, Illinois in 1991 was identified in Warren County, Illinois in 2013 (isolate K_ILSO2_3_25c) (Table 1). Interestingly, this MLG was also identified in Pickaway County and Morrow County in Ohio as well as in Merlin, Ontario (Table 1).

AMOVA (Table 3) showed significant population differentiation between the Illinois and Ontario populations for the uncensored data (Φ_{PT} = 0.167; P = 0.0007); however, after clone censoring, no genetic differentiation was observed between populations (Φ_{PT} = -0.005; P = 0.385). Variation between the Illinois and Ontario populations accounted for 0% of the total variance, whereas that within populations accounted for 100% (Table 3). From the estimates of the overall population genetic differentiation, F_{ST} , we found that, for the clone-censored data, 42.5 and 57.5% of the total variance were attributable to differences among individuals within populations and differences within individuals, respectively.

Reproductive mode and population structure. Significance testing of the estimates of I_A and $\bar{F}_d$ rejected the null hypothesis of sexual reproduction in the three populations for the

Fig. 2. Minimum spanning network showing the distribution and relationship between the multilocus genotypes (MLGs) observed in the five counties in Illinois where *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB was recovered. Each node/circle represents a different MLG, and the number inside each node represents the frequency of that MLG. Node color represents population membership. Connecting nodes are edges, wherein edge thickness and color are proportional to Nei's genetic distance indicated in the scale bar. Notations are as follows: Champaign County, IL-CH; DeKalb County, IL-DK; unknown county, IL-UN; Warren County, IL-WA; Williamson County, IL-WI; and Whiteside County, IL-WS.

TABLE 3. Analysis of molecular variance of *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB populations from Illinois and Ontario

Source of variation	Uncensored data				Clone-censored data			
	df	% of total	Fixation statistic	P value	df	% of total	Fixation statistic	P value
Φ_{PT}								
Among populations	1	16.70	0.167	0.0007	1	0	-0.005	0.415
Within populations	48	83.30			17	100		
F_{ST}								
Among populations	1	12.10	0.121	0.0001	1	0	-0.003	0.466
Among individuals within populations	48	32.81	0.373	0.0001	17	42.5	0.425	0.0001
Within individuals	50	55.09	0.449	0.0001	19	57.5	0.424	0.0001

noncensored data ($P < 0.0001$) and the Illinois and Ontario populations for the clone-censored data ($P < 0.0001$) (Table 2). Results from the bootstrapped trees showed that the three populations did not cluster according to geographic origin; rather, each cluster observed comprised members from each population. Interestingly, phylogenetic analysis revealed multifurcating branches with longer terminal branch lengths for most members

of the Ontario population, providing evidence for sexual reproduction in this population. Inbreeding coefficient values for Illinois, Ohio, and Ontario were 0.443 ($P = 0.0001$), 0.552 ($P = 0.005$), and 0.408 ($P < 0.0001$), respectively. DAPC clustering using uncensored data achieved a 95% overall reassignment of isolates to their prior groups; however, clone censoring reduced the overall reassignment to 79%. These findings

Fig. 3. Unweighted pair group method with arithmetic mean dendrogram of *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB isolates (uncensored data) from Illinois (IL), Ohio (OH), and Ontario (ON) based on Nei's genetic distance. Bootstrap support values >70% using 1,000 bootstrap samples are shown at the nodes.

indicate the presence of admixed genotypes, which were defined based on posterior membership probabilities <90%. For the uncensored data, 100, 86, and 93% of the genotypes making up the Illinois, Ohio, and Ontario populations, respectively, were assigned to their prior population. With clone censoring, membership probabilities dropped to 77, 50, and 88% for the Illinois, Ohio, and Ontario populations, respectively.

DISCUSSION

This work provides the first in-depth assessment of the genetic structure of *R. solani* AG-2-2IIIB populations associated with seedling disease of soybean in North America. Our first objective was to assess the genetic structure of this subgroup to determine if the observed diversity is consistent with that of a sexual or

Fig. 4. Unweighted pair group method with arithmetic mean dendrogram of *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB isolates (clone-censored data) from Illinois (IL), Ohio (OH), and Ontario (ON) based on Nei's genetic distance. Bootstrap support values >70% using 1,000 bootstrap samples are shown at the nodes.

recombining population. Given the lack of reports documenting the isolation of sexual structures in soybean fields as well as our unsuccessful attempts to induce sexual reproduction in vitro, we hypothesized that field populations were mostly clonal. One of the hallmarks of asexual reproduction is low genotypic diversity (McDonald and Linde 2002; Milgroom 1996), which is a function of the number of unique genotypes (genotypic richness) and their frequencies (genotypic evenness). Similarly, overrepresentation of a few MLGs and LD among loci are strong indicators of clonality (Tibayrenc et al. 1991). The results from this study suggest a mixed reproductive mode for the Ontario and Ohio populations and an asexual mode of reproduction for the Illinois population. A comparison of the eMLG, overall genotypic diversity, and clonal percentage revealed that the Ontario population was the most genotypically diverse. Despite the high genotypic diversity observed for the Ontario population, we found significant associations among alleles at different loci for both the uncensored and clone-censored data, providing support for predominantly clonal reproduction. However, phylogenetic analysis revealed long terminal multifurcating branches for most of the members of this population, which is typical of sexually outcrossing populations. These findings provide evidence to support a primarily asexual mode of reproduction with some sexual reproduction.

No previous studies have characterized the population genetic structure of *R. solani* AG-2-2IIIB from soybean-growing regions to allow for a comparison of genetic diversity or the role of sexual reproduction as a mechanism of genetic variation. However, a previous study found AG-2-2IIIB isolates recovered from turfgrass fields in Guelph, Ontario to be genetically diverse (Zheng et al. 2013). Similarly, Taheri and Tarighi (2012) found a high level of genetic diversity among AG-2-2IIIB isolates recovered from diseased sugar beet plants in Iran. Unfortunately, none of these studies reported the occurrence of sexual structures in the fields from which these isolates were recovered. Interestingly, the production of sexual spores in sugar beet fields by isolates of AG-2-2IV has been reported (Toda and Hyakumachi 2006). Also, on fields planted to table beet in New York, AG-2-2 isolates causing foliar blight and root rot have been found to produce the sexual

structures (Olaya and Abawi 1994). These observations altogether suggest a role for sexual reproduction as a source of genetic variation in AG-2-2, especially within the populations of the different subgroups.

Our rejection of the null hypothesis of sexual reproduction for the Ontario population may be because of several reasons. First, “founder effect” coupled with inbreeding might have had a significant impact. Inbreeding coefficient estimates from our results indicate that considerable inbreeding might be occurring in this population. If the population was started by very few related individuals with fixed differences in alleles, association among alleles will still be detected, although individuals within the population were randomly mating. Second, given the high dependence of LD on sample size, we may not have had enough statistical power to detect sexual recombination owing to the limited number of samples evaluated in this study. To better understand the role of sexual reproduction as a mechanism of variation in the populations of *R. solani* AG-2-2IIIB associated with soybean seedling disease, there is the need to continue searching for sexual reproductive structures in nature to corroborate inferences based on population genetics. Additionally, the role of heterokaryosis and mutation, both of which have been implicated as other possible mechanisms of variation in *R. solani* (Flentje and Stretton 1964), is also worth investigating.

Despite sample size differences between the Illinois ($n = 35$) and Ohio ($n = 7$) populations, genotypic richness was comparable between these two populations. However, an examination of the clonal fraction within each population revealed a higher clonal percentage for the Illinois population than for the Ohio population. Interestingly, we also found that MLGs identified in Illinois were shared among populations from different counties separated in time and space (Fig. 2). These findings coupled with our rejection of the null hypothesis of no LD among loci lend support to our hypothesis of a predominantly clonal population structure for the Illinois population. Differences in the genotypic diversities observed between the Illinois and Ontario populations may be explained by crop species diversity. Host diversity has been suggested as a potential factor influencing the genetic structure of fungal

Fig. 5. Discriminant analysis of principal components scatterplot showing the first and second principal components discriminating *Rhizoctonia solani* anastomosis group 2, subgroup 2IIIB populations from Illinois, Ohio, and Ontario. Lines are included to connect multilocus genotypes from the same population. **A**, Uncensored data. **B**, Clone-censored data. PCA, principal component analysis.

populations, including populations of *R. solani* AG-2-2IIIB (Taheri and Tarighi 2012) and those of *Magnaporthe grisea* (Douhan et al. 2011; Tredway et al. 2005). Although isolates making up the Illinois population were recovered from fields planted mostly to soybean and corn, fields from which the Ontario isolates were recovered are planted to a wide variety of crops, including soybean, corn, wheat, tobacco, vegetable crops, and fruit trees. In addition to host diversity as a possible explanation for the differences in genotypic diversity and reproductive mode, differences in fungal life cycles across different geographies may be an artifact of region-based differences in the biology and mode of dispersal of infective structures (Taylor et al. 1999).

A surprising finding from this study is the significantly low levels of population differentiation among the populations that are geographically separated. Partitioning of overall genetic variation among isolates of the Illinois and Ontario population revealed that 100% of the genetic variation was within populations and that 0% was between populations, suggesting no differentiation. Lack of population subdivision was further supported by phylogenetic analysis (UPGMA trees) (Figs. 3 and 4) and MLGs present across different geographies (populations) and counties within Illinois. Moreover, DAPC revealed the presence of admixed genotypes, especially after clone censoring (Fig. 5).

Our results are different from those reported for *R. solani* AG-2-2IIIB populations from sugar beet in Iran (Taheri and Tarighi 2012), which showed significant differentiation among isolates and no genotypes across geographic locations. However, results of other studies on *R. solani* AG-3 and AG-1A yielded results congruent with those presented here, showing lack of population differentiation and significant amounts of gene and genotype flow among geographically separated populations, which were attributed to human-mediated movement of infected plant material and movement of sclerotia via irrigation water (Bernardes-de-Assis et al. 2009; Ferrucho et al. 2013). We propose that the low level of genetic differentiation of populations in this study may be attributed to genotype flow via long-distance dispersal of asexual propagules, resulting in genetic similarity among isolates from different regions. Genotype flow may have occurred via anthropogenic activities that move soil, such as movement of contaminated vehicles and machinery, or via sclerotia dispersal, which may have been mediated by rainfall, irrigation, or drainage.

The importance of this study relates to how the information on the population genetic structure of *R. solani* AG-2-2IIIB can be exploited for improving management of the seedling disease that it incites on soybean. Genetic engineering for resistant host plants is limited for necrotrophic pathogens; consequently, there are no commercial soybean cultivars marketed as resistant to *Rhizoctonia* root and hypocotyl rot, which makes fungicide seed treatments the most common management method. Fungicide resistance development is of growing concern, with an overreliance on fungicide application as a sole management strategy. Although most of the fungicide seed treatment products used to control seedling diseases of soybean contain active ingredients with multiple modes of action, some products contain a single active ingredient belonging to a chemistry class that is at a high risk of selecting resistant genotypes. This is relevant, because mode of reproduction of the target pathogen affects the potential of a pathogen to evolve genotypes that are adapted to new environments and/or fungicide applications, where pathogens with mixed modes of reproduction are considered highest risk (McDonald and Linde 2002). This is because the process of asexual reproduction maintains and increases the frequency (via selection) of well-adapted genotypes created through the process of sexual reproduction. Our results indicate that populations of *R. solani* AG-2-2IIIB are characterized by either a predominantly mixed reproductive system or an asexual form of reproduction and that the exchange of genotypes among geographically separated populations is a predominant factor that shapes population structure. Therefore, there is the need to exercise

caution when applying fungicides and the need for the continued search for sources of resistance in the soybean germplasm to increase available options for disease management.

This study focused on populations of *R. solani* from two states in the United States and one province in Canada. Although overall sample size and the number of samples within each population examined may have been inadequate to make an overall assessment of diversity and detect random mating among members of each population, the low success rate associated with the recovery of *R. solani* isolates, especially those belonging to AG-2-2IIIB, from soybean roots is worth highlighting. Moreover, isolates that are most often recovered make up members of other AGs that cannot be included in population genetic structure analysis studies. There is, therefore, the need for more extensive sampling from similar and additional locations to improve the statistical power for detecting sexual recombination and clarify the role of gene and genotype flow as an evolutionary force shaping the population structure of this subgroup, especially on fields planted to soybean.

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