Genetic Structure of Populations of the Wheat Sharp Eyespot Pathogen *Rhizoctonia cerealis* Anastomosis Group D Subgroup I in China

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ABSTRACT

Sharp eyespot on wheat is caused by *Rhizoctonia cerealis* anastomosis group D subgroup I (AG-DI) and is an economically important stem-base disease of wheat in temperate regions worldwide. However, the understanding about the field population structure of *R. cerealis* is limited. In this study, the genetic structure of four wheat-infecting populations in China was investigated using six microsatellite markers characterized from the transcriptome data of *R. cerealis* AG-DI. A total of 173 unique genotypes were identified among 235 fungal isolates. Departure from Hardy-Weinberg equilibrium, a significant degree of inbreeding, and a significant deficit in heterozygotes indicated a nonrandom mating pattern. Combining the low to intermediate degrees of gametic disequilibrium, although with high genotypic

diversity and low to moderate clonal fractions, sexual reproduction probably existed, but the asexual reproduction should be the predominant reproductive mode. Structural analysis showed three gene pools among the four populations, which indicated the existence of three evolutionary origins of *R. cerealis* AG-DI. The long-distance movement of contaminated material, especially the infected seed, might have caused the moderate gene flow among these populations, which was consistent with the high differentiation among these populations. Overall, the genetic characteristics of the populations suggested a moderate evolutionary potential for *R. cerealis* AG-DI in China.

Additional keywords: population genetics.

Soilborne basidiomycetous fungus *Rhizoctonia cerealis* Van der Hoeven (teleomorph: *Ceratobasidium cereale* Murray and Burpee), which belongs to the binucleate *Rhizoctonia* anastomosis group D (AG-D), is the causal pathogen of foot rot, root rot, and damping-off diseases in several crops (González García et al. 2006; Lipps and Herr 1982). The AG-D subgroup I (AG-DI) can cause sharp eyespot on wheat and yellow patch on grasses (Hayakawa et al. 2006; Toda et al. 1999). The sharp eyespot caused by *R. cerealis* AG-DI is a stem-base wheat disease that is widely distributed in temperate wheat-growing regions worldwide (Hamada et al. 2011). In China, sharp eyespot has been a highly economically important wheat disease for many decades, especially in the lower reaches of the Yangtze River and the Yellow Huai River basins (Chen et al. 2009). In 2005, 8.1 million ha of winter wheat was affected by *R. cerealis*, demonstrating the importance and urgency of preventing and controlling this disease (McBeath and McBeath 2010). Wheat cultivars resistant to sharp eyespot can be used to prevent this disease. However, few wheat cultivars demonstrate moderate resistance, and highly resistant cultivars are rare (Chen et al. 2013). Fungicide seed coating and spraying at early jointing stage remain the main control strategies, despite the problem of increasing fungicide residues in crops and in the environment. The potential risk of the emergence of pathogen populations resistant to fungicides is also the concern, although no resistant isolates of *R. cerealis* have been detected in the field until now (Sun et al. 2015).

R. cerealis AG-DI does not form asexual spores, and its sexual stage is probably rare in nature (González García et al. 2006; Hayakawa et al. 2006). This fungus exists over summer or winter as a vegetative mycelium or sclerotium in plant debris or soil. Mycelium or germinating sclerotium serves as a primary inoculum

and infects wheat before winter or the coming spring (Hamada et al. 2011; Lipps and Herr 1982). By using rDNA-internal transcribed spacer (ITS) sequences, preliminary studies have provided evidence for the heterogeneity of the ITS sequences and the evolution of the rDNA of *R. cerealis* AG-DI in China (Li et al. 2014).

Despite the high yield losses caused by *R. cerealis* on wheat globally, the population biology of this fungus remains poorly understood. The population genetics of multinucleate *R. solani* (teleomorph: *Thanatephorus cucumeris*) has recently been studied using the microsatellite markers developed from enriched genomic libraries (Zala et al. 2008) or draft genome (Ferrucho et al. 2009). Among the different strains investigated were the rice-, soybean-, maize-, and signalgrass-infecting *R. solani* AG-1 IA (Bernardes-de-Assis et al. 2008, 2009; Chavarro Mesa et al. 2015; Ciampi et al. 2008; Padasht-Dehkaei et al. 2013); the potato-infecting *R. solani* AG-3 (Ferrucho et al. 2013); and the vegetable-infecting *R. solani* AG-4 (Haratian et al. 2013). For the binucleate *Rhizoctonia*, only the population genetic information on *R. oryzae-sativae* (teleomorph: *C. oryzae-sativae*, under AG-B), which is the causative pathogen of the rice aggregate sheath spot in California and Latin America, is available to date (Chaijuckam et al. 2010; Pereira et al. 2016).

No polymorphic microsatellite markers are currently available for population genetic studies with *R. cerealis*. In pathogenic fungi, microsatellites or tandem simple-sequence repeat (SSR) markers become powerful tools for population structure studies, particularly in understanding the evolutionary potential of populations and making predictions regarding the relative risks posed by pathogens in terms of fungicidal applications and breakdown of resistance genes (McDonald and Linde 2002). The present study aimed to (i) develop a repertoire of primer pairs that can amplify polymorphic microsatellite loci in *R. cerealis* AG-DI and employ these SSR primers to analyze the population genetics of *R. cerealis* AG-DI populations in China, (ii) measure the genetic and genotypic diversity in *R. cerealis* AG-DI populations infecting wheat, (iii) infer the predominant reproductive mode in these populations,

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and (iv) determine the occurrence and the magnitude of gene flow among populations.

MATERIALS AND METHODS

Fungal isolates. In 2010, wheat samples showing typical symptoms of sharp eyespot were collected from four different fields in China, namely, Bengbu in the Anhui Province (BB; 32.55°N, 120.21°E), Linyi in the Shandong Province (LY; 34.88°N, 118.17°E), Jiangyan in the Jiangsu Province (JY; 34.25°N, 116.21°E), and Shangqiu in the Henan Province (SQ; 33.04°N, 117.38°E). From each field, a total of 50 to 80 infected samples were collected. These samples were separated from one another by at least 10 m along each transect, and seven to nine transects per field were carried out. The infected stems were cut into 5- to 10-mm-long segments; processed with 75% alcohol, NaOCl (1% available Cl), and sterile water; placed on petri plates containing potato dextrose agar (PDA) medium; and then incubated at 25°C in the dark. After 2 to 3 days of incubation, hyphal tips from the colonies of *R. cerealis* AG-DI were transferred to fresh PDA plates. From each field population, 51 to 70 isolates were obtained. To determine the AG of the isolates, a pair of primers developed from the *tubB* gene of *R. cerealis* AG-DI were used to amplify specific segments: RtubF4 (5'-CCT AAA TGA GTC TGG AGT AAG TC-3') and RtubR4 (5'-GCT AGT GCG GTC AAT GTA TAG-3') (Guo et al. 2012).

Microsatellite markers. In our previous study, we obtained the transcriptome (mRNA) of *R. cerealis* AG-DI strain R0301, which contained 21,029 unigenes with lengths of 200 to 15,995 bp (Ren et al. 2013) (W. Li, unpublished data). The unigene sequences were screened for microsatellites by using Magellan software (Lim et al. 2004). All possible mono- to hexa-nucleotide motifs of five or more repeats in the transcriptome database were identified. Magellan was also designed to list the locations of all microsatellites found in each unigene sequence, thereby allowing easy-to-design primers to amplify selected microsatellite loci based on the surrounding sequences. The primer pairs were designed using Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, CA) and synthesized by Genscript (Genscript Biotech, Nanjing, China).

To test the polymorphism of the microsatellite markers, 15 *R. cerealis* AG-DI isolates from several areas of China were randomly selected. Polymerase chain reactions (PCRs) were performed in a total of 20 µl volumes with 1 µl of genomic DNA (5 to 10 ng of final concentration), 5 µl of 10× reaction buffer (500 mM KCl, 15 mM MgCl₂, 100 mM Tris-HCl, pH 8.8), 0.3 µM of each primer, 2 µl of dNTP mixture (each 2.5 mM), and 1 U of *Taq* polymerase (TaKaRa). Reactions were conducted in an S1000 TM thermal cycler (Bio-Rad) programmed for an initial incubation of 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C or 62°C for 30 s, and 72°C for 30 s. A final extension reaction was conducted at 72°C for 10 min. Amplified products were analyzed by electrophoresis on 2% agarose and 8% polyacrylamide gels. Some of the PCR products amplified using high-fidelity polymerase (PrimeSTAR HS DNA Polymerase, TaKaRa) were ligated with pSIMLPE-18 *EcoRV*/BAP vector (TaKaRa), amplified in *Escherichia coli* JM109, and sequenced in Genscript (Genscript Biotech) to ensure the accuracy of the type and repeat number of the motifs.

The forward primers of the final microsatellite loci were labeled with 6-carboxyfluorescein (6-FAM) fluorescent dyes (Genscript Biotech). PCR products were separated on an ABI3730 Genetic Analyzer by using GeneScan 600 LIZ as a size standard (Applied Biosystems). The size of the fragments for each SSR was determined using GeneMapper software (Applied Biosystems), adjusted manually if necessary, and reported in Excel.

Microsatellite data analyses. *R. cerealis* AG-DI is a dikaryon (Li et al. 2014); thus, we assumed that the genetic conditions of all of the isolates used in this study were similar to a diploid organism. Data on the range of repeats of each locus, number of alleles per

locus, identification of private alleles, and allelic frequencies were assessed using CONVERT version 1.31 (Glaubitz 2004).

Genotype diversity. The program GenoType (Meirmans and Van Tienderen 2004) was used to determine the multilocus microsatellite genotype (MLMG) of each strain. Isolates with the same MLMG were treated as clones. Several indices of clonal diversity were estimated for each population by using GenoDive (Meirmans and Van Tienderen 2004). These indices included (i) the number of genotypes per population; (ii) the clonal fraction (or the proportion of fungal isolates originating from asexual reproduction), calculated as $1 - [(number\ of\ different\ genotypes)/(total\ number\ of\ isolates)]$ (Zhan et al. 2003); and (iii) both Stoddart and Taylor's genotypic diversity and its evenness. Stoddart and Taylor's genotypic diversity is expressed as $G_0 = 1/\sum p_i^2$, where p_i is the frequency of the i th genotype (Stoddart and Taylor 1988), and its evenness is expressed as G_0 scaled by the maximum number of expected genotypes. The latter is an indicator of how evenly the genotypes are distributed over the population. By using a pairwise bootstrap test, we calculated whether pairs of populations differed in their clonal diversity indices. In this process, the individuals were resampled from the populations, and the diversity indices were compared after every replicate (Manly 1991). A total of 1,000 permutations with subsampling were employed to match the size of the smallest population (Grünwald et al. 2003). For all subsequent analyses, clone-corrected datasets were used.

Genetic diversity and differentiation among geographical populations. Genetic diversity was quantified on the basis of allelic richness and expected heterozygosity (HE) using FSTAT 2.9.3 (Goudet 1995). Allelic richness was estimated in accordance with the method of El Mousadik and Petit (1996) to determine the mean number of alleles per locus. HE was estimated in accordance with Nei's formula as follows: $n/(n-1) \times (1 - \sum p_i^2)$, where p is the observed frequency of the i th allele and n is the sample size (Nei 1978). A bootstrapping approach based on 1,000 permutations was also performed using FSTAT 2.9.3 (Goudet 1995) to test whether the populations differed in genetic diversity and allelic richness.

The degree of population subdivision and the distribution of genetic diversity among populations of *R. cerealis* AG-DI were estimated by pairwise comparisons of populations through analysis of molecular variance (AMOVA) with the program Arlequin 3.5 (Excoffier et al. 2005). A hierarchical analysis of variance was used to partition the covariance components into those among populations and those within populations. The pairwise fixation index (R_{ST}) between groups of populations was calculated to examine population differentiation. The significance of both analyses was determined using 1,000 permutations by a nonparametric approach in Arlequin 3.5 (Excoffier et al. 2005).

Reproductive mode: Hardy-Weinberg equilibrium (HWE) and gametic equilibrium (GE) tests. HWE analysis was performed using Fisher's exact test with a Markov chain (chain length: 1,000,000; dememorization: 100,000) by employing Arlequin 3.5 (Excoffier et al. 2005). GE was assessed using a multilocus association test (Brown et al. 1980). A hypothesis proposing that genotypes at one locus are independent from genotypes at another locus was evaluated using the Markov chain Monte Carlo (MCMC) algorithm (with 1,000 batches and 1,000 iterations per batch), which was implemented in GenePop 4.0.11 (<http://genepop.curtin.edu.au/>) (Raymond and Rousset 1995). Using Multilocus v1.3 (Agapow and Burt 2001), we measured the multilocus index of association (I_A) (Smith et al. 1993). The inbreeding coefficient R_{IS} was calculated using Arlequin 3.5 (Excoffier et al. 2005) with 1,000 permutations of the dataset.

Test for admixture or hidden population structure. The admixture of the population is a possible cause of observed departures from HWE and GE. A clustering method based on the Bayesian model was implemented in Structure 2.3.4 to test the pattern of population structure and detection of probable introgression (Pritchard et al. 2000). In this process, an admixture model with correlated allele frequencies was adopted. To achieve the optimum K value, five replicates were run, 10,000 burn-in

periods were employed, and 100,000 MCMC replications for data collection were conducted (Falush et al. 2003). The optimum number of clusters (ΔK) was calculated between $K = 1$ and $K = 7$, in accordance with the simulation method of Evanno et al. (2005) using Structure Harvester v0.6.8 (https://users.soe.ucsc.edu/~dear/structure/struct_harvest/) (Earl 2012). To facilitate interpretation, CLUMPP (<http://web.stanford.edu/group/rosenberglab/clumpp.html>) (Jakobsson and Rosenberg 2007) was used to align multiple replicate analyses of the dataset from Structure Harvester.

Historical migration. The effective population sizes (θ) and historical migration rates ($4Nm$) among the four populations were estimated using a maximum likelihood test with MIGRATE 3.2 (Beerli and Felsenstein 2001). MIGRATE is based on a coalescent theory to estimate θ values and the migration matrix (Beerli and Felsenstein 2001). This program can be used to infer the asymmetrical gene flow among populations by analyzing the microsatellite data with Brownian motion and assuming a stepwise mutation model. A single run consisted of five replicates with 10 initial short chains and 3 long final chains, as well as a static heating scheme with four temperatures (1.0, 1.3, 2.6, and 3.9). For the short chain, the recorded steps were 1,000, the increment was 20, and the visited genealogies were 20,000. For the long chain, the recorded steps were 5,000, the increment was 20, and the visited genealogies were 100,000. The number of discarded trees per short and long chains was 10,000. The confidence interval for θ and migration parameter M was calculated using a percentile approach.

RESULTS

Microsatellite markers and fungal populations. Among 21,029 unigene sequences, we found 220 di-, 112 tri-, and 1 hexa-nucleotide microsatellite motifs (Table 1). No tetra- and penta-nucleotide motifs were found in these sequences (Table 1). However, most of the microsatellite loci were located in the 5' or 3' end of the unigene sequences, on which no primers can be designed. Given these sequences, 24 microsatellite loci, comprising seven di-, nine tri-, one hexa-, and seven compound-nucleotide

TABLE 1. Number of microsatellite motifs in the transcriptome of *Rhizoctonia cerealis* anastomosis group D subgroup I

Motif	Number of repeats						Sum of motifs
	5	6	7	8	9	>10	
Mono	35,472	7,937	1,485	276	89	114	45,373
Di	189	27	3	1	0	0	220
Tri	85	17	5	3	0	2	112
Tetra	0	0	0	0	0	0	0
Penta	0	0	0	0	0	0	0
Hexa	0	0	0	1	0	0	1

microsatellite motifs, were identified and selected to develop primer pairs. Among these microsatellite loci, 17 were successfully amplified from the *R. cerealis* AG-DI strains, and 11 exhibited a size polymorphism within different strains. To ensure the accuracy of the type and repeat number of the motifs, these 11 microsatellite loci were amplified using a high-fidelity polymerase, and the PCR products were sequenced and analyzed. Finally, six microsatellite loci were confirmed, of which two were dinucleotide motifs with 2 to 12 repeat numbers, three were trinucleotide motifs with 3 to 12 repeat numbers, and 1 was a hexa-nucleotide motif with one to eight repeat numbers. The primer sequences and the allele size range are listed in Table 2.

A total of 235 isolates from four geographical populations were analyzed in this study, of which 57 isolates were from the BB populations, 70 from the LY, 57 from the JY, and 51 from the SQ (Table 3). All isolates were identified to be *R. cerealis* AG-DI on the basis of morphological characteristics (Chen et al. 2009) and specific primer amplification RtubF/R4 (Guo et al. 2012). All the loci were polymorphic in the four populations of *R. cerealis* AG-DI with high genetic diversity (Table 2). Two to four private alleles were found in the BB, LY, and JY populations, whereas no private alleles were found in the SQ population (Table 3).

Microsatellite information content and genetic and genotypic diversity. A total of 173 different genotypes were detected among the four populations of *R. cerealis* AG-DI. Overall, only two genotypes were shared between the BB and LY populations. Small to intermediate clonal fractions were noted for all populations. The smallest clonal fraction was 0.12 in the SQ population, which exhibited the highest genotypic diversity (37.7) and evenly distributed genotypes (evenness = 0.84) (Table 3). Intermediate clonal fractions (0.21 to 0.41) were observed for the other three populations. LY, which is the most northern population, displayed the highest clonal fractions (0.41) and site-specific genotypes (39) and exhibited the lowest genotypic diversity (19.6) and evenness (0.48) (Table 3). The majority of the genotypes (171 of the 173) were heterozygous for at least one locus. The HE was 0.51 to 0.59, and Nei's genetic diversity values were not significantly different ($P \leq 0.05$) among the four populations (Table 3).

Population differentiation. On the basis of the AMOVA estimates, significant differentiation existed among the populations. The variations among populations only represented 3.69% of the total variance. By contrast, the variation within individuals accounted for 69.13% of the total variance (Table 4). Pairwise analysis of population differentiation also indicated that these four populations were significantly differentiated, except the differentiation between the BB and LY populations ($R_{ST} = 0.008$, $P = 0.12$, insignificant). The highest R_{ST} value was found between the BB and JY populations ($R_{ST} = 0.064$, $P = 0.0$), and the other R_{ST} values were 0.038 to 0.045 ($P = 0.0$) (Table 5).

TABLE 2. Description of primers, conditions of annealing temperature for PCR amplification, allele size, and number of repeats

Marker name	Repeat motif		Primers (5'-3')	Tm (°C)	Allele size range (bp)	Number of alleles	Gene diversity ^z			
							BB	LY	JY	SQ
R4	(AGA)5-9	R4-u	AGTAGAGGTGATGGAGGAAG	58	275–287	5	0.69	0.73	0.69	0.71
		R4-d	CTGATGACGCTGTATGGTAT							
R5	(GCA)3-6	R5-u	AGCAGCAGATGCAGATGCAG	62	415–424	4	0.33	0.12	0.10	0.18
		R5-d	GTCCCAACAACGCTCCCTCAT							
R14	(GTG)4-12	R14-u	CATCCCGTTGTGTGCCATTG	62	315–339	9	0.82	0.76	0.67	0.85
		R14-d	GTCTCTCTCACCACCGCAGT							
R15	(CGGAAA)1-8	R15-u	TTGTTGAGCGCTTGAGCCAG	62	214–256	8	0.64	0.67	0.51	0.27
		R15-d	CTCGCCGTCCTTCGATAAAC							
R21	(TA)2-11	R21-u	ATTACGGTGACGGATGGT	58	340–358	9	0.28	0.38	0.41	0.38
		R21-d	CTCCAGGCGTTACGAATA							
R24	(TG)4-12	R24-u	GCTACTAGGGTCCTTTCA	58	308–324	9	0.44	0.52	0.72	0.73
		R24-d	AAACCAACGGAGTTACGG							

^z Unbiased gene diversity was computed according to Nei (1978) using FSTAT version 2.9.3.

HWE and GE tests. The BB population exhibited half of the loci in the HWE, whereas the other populations contained more than half loci out of HWE (Table 6). All four populations of *R. cerealis* AG-DI showed significant departures from HWE. Positive values of inbreeding coefficients R_{IS} from 0.11 to 0.31 ($P \leq 0.01$) were obtained for the four populations, and this finding indicated the significant inbreeding in these populations. In the GE test, the BB ($I_A = 0.071$, $P = 0.208$) and LY ($I_A = 0.093$, $P = 0.147$) populations were in GE. Significant I_A was observed in the JY ($I_A = 0.147$, $P = 0.025$) and SQ ($I_A = 0.151$, $P = 0.032$) populations. However, 20 to 40% of locus pairs were in disequilibrium.

Test for admixture or hidden population structure. By using Structure Harvester and on the basis of ΔK value, we analyzed the optimum number of clusters. The results from Structure Harvester indicated that the highest peak of ΔK appeared when $K = 3$ (Evanno et al. 2005). Given this result, we considered $K = 3$ to be the supported number of populations. Subsequently, three were selected as the optimal number of groups. The subpopulation structure in 173 lines was determined as three different bar plots (Fig. 1). The diagram showing the individual assignments to the clusters revealed a clonal structure to the data. In the three-cluster model ($K = 3$), varying shades of gray represent three gene pools. All of the populations exhibited a similar cluster, with mixed individuals.

Demographic parameters and historical migration. The effective population size estimates of the BB, JY, and SQ populations were highly similar ($\theta = 0.93$, 0.91, and 0.94), whereas that of the LY population was larger ($\theta = 1.16$) (Fig. 2). The historical migration rates provide evidence of directional gene flow among the different populations. In this study, evidence for gene flow was found among all *R. cerealis* AG-DI populations in China. Increased migration was found between the two northern populations ($4Nm_{LY \rightarrow SQ} = 1.59$, $4Nm_{SQ \rightarrow LY} = 2.72$). Most of the exchanges of immigrants between these populations were asymmetrical. The highest asymmetric migration estimates were observed between LY and BB ($4Nm_{LY \rightarrow BB} = 3.33$, $4Nm_{BB \rightarrow LY} = 1.11$). The population that contributed the highest number of migrants to majority of the populations (except JY) was LY ($4Nm_{LY \rightarrow BB} = 3.33$, $4Nm_{LY \rightarrow SQ} =$

1.59) (Fig. 2). Generally, all the populations contributed migrants to other populations, and the northern populations (LY and SQ) contributed more than the southern populations did (BB and JY).

DISCUSSION

In this study, we analyzed all the microsatellite loci in the transcriptome of the *R. cerealis* AG-DI strain R0301. We also described a set of primer pairs that can amplify six polymorphic microsatellite loci in *R. cerealis* AG-DI. We used these markers to analyze four populations comprising a total of 235 isolates from China. The results indicated that the microsatellite markers developed in this study were species-specific and can be used to distinguish a large number of *R. cerealis* AG-DI genotypes. To our knowledge, this paper reports the first codominant microsatellite markers of *R. cerealis*. Therefore, this set of microsatellite markers may represent an important tool for population genetic investigations on this fungus.

R. cerealis AG-DI is mainly considered as an asexual fungus on wheat. Sclerotia are produced at the end of the season and infect the next wheat crop, but the sexual stage of this fungus remains unknown (González García et al. 2006; Hayakawa et al. 2006; Lipps and Herr 1982; Toda et al. 1999). Mixed reproductive modes were

TABLE 5. Fixation indices (R_{ST}) for pairwise comparisons between *Rhizoctonia cerealis* anastomosis group D subgroup I populations^z

Population	Sum of squared size differences (R_{ST})			
	BB	LY	JY	SQ
BB	—			
LY	0.008 ^{NS}	—		
JY	0.064*	0.042*	—	
SQ	0.038*	0.045*	0.045*	—

^z Pairwise fixation indices were computed between pairs of populations with 1,000 using Arlequin 3.5 (Excoffier et al. 2005). NS and * indicate insignificant or significant at $\alpha = 0.05$.

TABLE 3. Measures of genotypic diversity in *Rhizoctonia cerealis* anastomosis group D subgroup I populations

Population	Sample size	Number of genotypes	Site-specific genotypes ^s	Clonal fraction	Effective number of genotypes ^{t,u}	Evenness ^{u,v}	HE ^{w,x}	Allelic richness ^{x,y}	Number of private alleles ^z
BB	57	42	40 (2)	0.26	31.54 a	0.75 a	0.55 a	5.33 a	2
LY	70	41	39 (2)	0.41	19.60 b	0.48 b	0.59 a	6.17 a	3
JY	57	45	45 (0)	0.21	35.70 ac	0.79 a	0.53 b	5.26 a	4
SQ	51	45	45 (0)	0.12	37.70 c	0.84 a	0.51 b	4.83 a	0
Total	235	173	Overall =	0.25	31.1	0.72	—	5.40	—

^s Numbers of genotypes shared with other populations are shown in parentheses.

^t Stoddart and Taylor's genotypic diversity (G_0) (Stoddart and Taylor 1988).

^u Means followed by the same letter are not significantly different (at $P \leq 0.05$) under pairwise bootstrap test for differences in clonal diversity indices between populations calculated by GenoDive (Meirmans and Van Tienderen 2004), using 1,000 permutations with subsampling to match the size of the smallest population.

^v G_0 scaled by the maximum number of expected genotypes; evenness value = 1.0 indicates that all genotypes possess equal frequencies.

^w Nei's unbiased gene diversity, also known as expected heterozygosity, averaged over all loci, corrected for the sample size (Nei 1978).

^x To test whether pairwise samples differed for allelic richness, we used FSTAT version 2.9.3 (Goudet 1995), on the basis of 1,000 permutations; means followed by the same letter are not significantly different ($P \leq 0.05$).

^y Calculated according to El Mousadik and Petit (1996).

^z Alleles occurring in only one population, calculated with Convert 1.31 (Glaubitz 2004).

TABLE 4. Hierarchical distribution of gene diversity among *Rhizoctonia cerealis* anastomosis group D subgroup I populations^z

Source of variation	Distance method: sum of squared size differences (R_{ST})				
	Sum of squares	Variance components	Percent variation	Fixation indices	P
Among populations	22.38	0.06	3.69	$R_{IS} = 0.28$	0.01*
Among individuals within populations	351.15	0.46	27.18	$R_{ST} = 0.04$	0.02*
Within individuals	201.50	1.17	69.13	$R_{IT} = 0.31$	0.06
Total	575.03	1.69			

^z Analysis of molecular variance was performed using Arlequin 3.5 (Excoffier et al. 2005). * indicates significant at $\alpha = 0.05$.

reported for *R. solani* AG-1 IA, AG-3, AG-4, and *R. oryzae-sativae* (Bernardes-de-Assis et al. 2008, 2009; Chaijuckam et al. 2010; Ciampi et al. 2008; Ferrucho et al. 2013; Haratian et al. 2013; Padasht-Dehkaei et al. 2013). An increasing number of studies have documented that basidiospores may play an important role in the disease cycle and thus cause important ecological and population consequences for the *Rhizoctonia* fungi (Bernardes-de-Assis et al. 2008, 2009; Chaijuckam et al. 2010; Ciampi et al. 2008; Cubeta and Vilgalys 1997; Ferrucho

et al. 2013; Haratian et al. 2013; Padasht-Dehkaei et al. 2013). In this study, the clonal fractions of *R. cerealis* AG-DI at 0.12 to 0.41 were unexpected results (Table 3), but most of the loci departed from HWE, with a significant degree of inbreeding, and half of the populations were in GD (Table 6). Hence, asexual reproduction may be the predominant reproduction model in *R. cerealis* AG-DI.

Sexual crossing generally causes genomic recombination in fungi (McDonald and Linde 2002; Milgroom 1996). In the asexual

TABLE 6. Hardy-Weinberg equilibrium (HWE) and gametic disequilibrium (GD) tests for *Rhizoctonia cerealis* anastomosis group D subgroup I populations

Population	Number of genotypes	Number of loci under HWE ^w	R_{IS}^x	Gametic equilibrium estimates					
				I_A^y	P value ^y	Locus pairs at significant disequilibrium ^z			%
BB	42	3/6	0.113	0.071	0.208	4/15			26.7
LY	41	1/6	0.307	0.093	0.147	5/15			33.3
JY	45	2/6	0.314	0.147	0.025*	6/15			40
SQ	45	2/6	0.203	0.151	0.032*	3/15			20

^w HWE test was performed by Fisher's exact test using a Markov chain (chain length: 1,000,000; dememorization: 100,000) in Arlequin 3.5.
^x Population-specific inbreeding coefficients (R_{IS}) and P values were calculated with 1,000 permutations using Arlequin 3.5.
^y Index of association (I_A) and P values were computed in Multilocus v1.3 (Agapow and Burt 2001); the significance of I_A was tested by comparing the observed and expected values under the null hypothesis of random mating with 1,000 randomizations. * indicates significant at $\alpha = 0.05$.
^z Pairs of loci at significant disequilibrium in accordance with the Fisher exact test (probability test) using a Markov chain with 1,000 batches and 1,000 iterations/batch, implemented by GenePop 4.0, after Bonferroni correction for multiple comparisons.

Fig. 1. Structure-inferred membership coefficient for multilocus microsatellite genotypes of the populations of *Rhizoctonia cerealis* anastomosis group D subgroup I in China. Each individual is represented by a thin vertical bar, which is partitioned into K shaded segments that represent the estimated membership fractions of an individual in $K = 3$ clusters. Black lines separate individuals of different populations. The bar length indicates its membership coefficient to the distinctly shaded populations.

Fig. 2. Estimates of demographic parameters from the divergence among wheat-infecting populations of *Rhizoctonia cerealis* anastomosis group D subgroup I in China. The directions for migration between the sink and source populations are indicated by the arrows, and the migration rate per generation ($4Nm$) is displayed on the arrows. Estimates of the theta include 95% confidence intervals indicated as vertical bars.

reproduction model of *R. cerealis* AG-DI, whether recombination will occur is currently unknown. By contrast, hyphal anastomosis is a common phenomenon among isolates of the same AG in the genus *Rhizoctonia*. Previous studies have shown the existence of two phenotypes (C2 and C3) of hyphal anastomosing reactions among different isolates belonging to the same AG (Cubeta and Vilgalys 1997). C2 involves a killing reaction and evident wall fusion, with cell death of anastomosing and adjacent cells. In this case, the somatic incompatibility response is often macroscopically visible. By contrast, C3 includes perfect fusion, with evident wall and membrane merging. The point of anastomosis is not clearly visible, and cell death is absent as these two phenotypes of interactions (C2 and C3) represent different genetic relationships among isolates (Cubeta and Vilgalys 1997; González García et al. 2006). If genomic recombination can occur through hyphal fusion in this fungus, the phenomenon should be nonrandom mating, consistent with the significant degree of inbreeding in *R. cerealis* AG-DI (Table 6). To our knowledge, no previous study has demonstrated the transmission of cytoplasmic element and nucleus via hyphal anastomosis in *Rhizoctonia* fungi (Cubeta and Vilgalys 1997). However, this probability cannot be ruled out, and the transfer of genetic material via hyphal anastomosis remains a possibility. This hypothesis must be further investigated and confirmed in the laboratory.

Population genetic analysis provided the resolution to infer the life histories and the evolutionary process of pathogens that shape pathogen populations in agroecosystems (McDonald and Linde 2002). Our previous study obtained 36 unique ITS sequences from 15 *R. cerealis* AG-DI isolates (Li et al. 2014). According to the phylogeny analysis and median-joining network, all these sequences can be divided into three groups, which implied the possibility of three evolutionary origins of this fungus (Li et al. 2014). In the current study, structure analysis showed that all of the 173 unique genotypes can be separated into three groups (Fig. 1). Although the isolates are different in the two studies, the present results suggested the existence of three gene pools in *R. cerealis*. The fact that four populations from different locations were divided to three groups, might indicate that geographic isolation did not cause reproductive isolation. And another possibility is that three evolutionary origins of this fungus existed before the geographic isolation of *R. cerealis*.

The genotypic flow in *R. solani* AG-1 IA, the causal agent of sheath blight disease in rice, is potentially mediated through the dispersal of sclerotia in irrigation water. In particular, high historical migration rates are found among rice-infecting populations from the Yangtze tributary region in China (Bernardes-de-Assis et al. 2009). For *R. cerealis* AG-DI, the sclerotia are the most important organs in summer and winter, serving as the primary inocula. This component is found between the leaf sheath and stem or within the stem lumen of the wheat. The sclerotia of *R. cerealis* AG-DI typically cannot travel long distances via irrigation water, especially in northern China. However, high historical migration rates (maximum $4Nm = 3.33$) are also noted in these populations. In the current study, increased genetic exchange existed between the two northern populations (LY and SQ), and they contributed more migrants than the southern populations (BB and JY) did (Fig. 2). We hypothesized that the genetic flow in this area can be mainly ascribed to the human-mediated long-distance movement of the contaminated materials, such as infested seed or straw, from north to south. Considering that winter wheat is the major cultivar in northern China, similar cultivars can also be planted sometimes in the Yangtze River Basin. Thus, the pathogen can spread with the infested seed to southern areas. However, most of the southern wheat cultivars are spring wheat, and they cannot be planted in northern areas. The spread of the contaminated material through the harvester is another possible reason, but the harvest of wheat in China occurs from south to north. Compared with the spread by infested seed, this hypothesis was not significant. Thus, the seed treatment with fungicides would be useful to prevent long-distance dispersal in these areas.

This paper presents the first in-depth population genetic analysis of the wheat sharp eyespot pathogen *R. cerealis* AG-DI from China. In these populations, we found high genetic and genotypic diversity, moderate gene flow, and asexual reproduction as the predominant reproductive model with significant inbreeding. Medium to high differentiation among populations was found based on pairwise analyses of population differentiation. According to the risk model framework proposed by McDonald and Linde (2002), the population genetic characteristics of *R. cerealis* AG-DI in China indicated that this pathogen demonstrated a moderate evolutionary potential. Hence, disease management measures such as traditional fungicides and the moderate resistant wheat cultivars could still be used. At the same time, the new fungicide development and breeding new resistant varieties also need attention.

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