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Genetic diversity and differentiation of *Sclerotinia sclerotiorum* populations in sunflower

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Abstract Ninety-six isolates of sunflower *Sclerotinia sclerotiorum* (Lib.) de Bary from Inner Mongolia (IM) in China, from Canada and the United Kingdom (UK) were sampled to investigate the genetic diversity and structure using Sequence-Related Amplified Polymorphism. A total of 123 polymorphic bands were obtained, ranging in size from 100 to 500 base pairs. The five populations of *S. sclerotiorum* isolated from the three countries showed various levels of genetic variability. The percentage of polymorphic loci varied from 30.89% in the UK population to 97.56% in the Middle IM population. The values of Shannon index (*i*) varied from 0.1876 in the UK population to 0.5301 in the West IM population. The heterozygosity of the five geographic populations obtained by estimating allele frequency varied from 12.91% in the UK population to 35.44% in the West IM population. The genetic identity, as indicated by the Nei unbiased identity index, ranged from 0.9744 between populations from Canada and East IM to 0.6477 between populations from West IM and UK. UPGMA cluster analysis using Nei's genetic distance gave distances ranging from 0.0259 to 0.4343. The rates of gene flow among five geographic populations ranged from 1.5406 between West IM and UK populations to 18.4149 between West IM and Middle IM populations. The four populations from West IM, Middle IM, East IM and Canada were clustered into one subgroup in which the isolates from West and Middle IM belonged to one population, whereas those from East IM and Canada essentially were another population. The isolates from the UK formed a population that was significantly distinct from other populations.

Keywords *Sclerotinia sclerotiorum* · Sequence-related amplified polymorphism · Genetic diversity · Population structure

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Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is one of the most devastating and cosmopolitan plant pathogens. The fungus infects over 400 species of plants

worldwide including important crops and numerous weeds (Boland and Hall 1994). *S. sclerotiorum* poses a threat to dicotyledonous crops such as sunflower, soybean, oilseed rape and various vegetables, but also monocotyledonous species such as onion and tulip (Boland and Hall 1994).

Sunflower is a main cash crop from which one third of farmers' income is obtained in Inner Mongolia (IM) Autonomous Region, China. The production area of sunflower in IM is approximately half a million hectares that consist of three major production regions: West IM, Middle IM—that is approximately 300 km from West IM, and East IM, approximately 2,500 km from Middle IM. In IM, *S. sclerotiorum* has caused serious head and stem rots in sunflower. A previous survey in 2004 showed that 85% of sunflower fields were infected and 25% to 30% of sunflower plants in the field were infected by *S. sclerotiorum* in IM (Li 2004). Therefore, the sunflower industry, local economy and income of farmers have been seriously limited by this disease in recent years.

Generally, it is important to understand the epidemiology and genetic diversity of the pathogen population regionally to control plant diseases by fungicides or resistant cultivars. Genetic analyses have shown that isolates of *S. sclerotiorum* are homogenous with limited variability in the 18S and 28S rDNA regions (Kohli et al. 1995). Previous studies have demonstrated that *S. sclerotiorum* populations in canola in Canada and cabbage in the United States are clonal. Clonal lineages have also been isolated from Ontario and Quebec from *Sclerotinia* strains isolated from soybean (Hambleton et al. 2002). However, isolates could be separated into distinct mycelial compatibility groups (MCGs) (Cubeta et al. 1997; Kohn et al. 1991). Individual isolates of *S. sclerotiorum* are also classified into clonal lineages by the use of two or more independent markers such as MCGs and microsatellites (Auclair et al. 2004; Carbone et al. 1999; González et al. 1998; Sirjusingh and Kohn 2001). However, MCGs or microsatellite markers have not been associated with specific virulence characteristics or ecological adaptations of the pathogen. A lack of variation in virulence among isolates from defined geographical areas has been reported in a number of studies on agricultural populations (Alvarez and Molina 2000; Atallah et al. 2004; Kull et al. 2004; Sexton and Howlett 2004). Differences in virulence may be detected when comparing isolates from widely separated geographical regions, but there has been no conclusive evidence to suggest host specialization among isolates of *S. sclerotiorum* (Kull et al. 2004).

A number of molecular techniques have been used to investigate genetic diversity of pathogen populations (White et al. 1990; Woo et al. 1998; Wright 1978) in plants. Among them, amplified fragment length polymorphism (AFLP) seems to be a more effective polymerase chain reaction (PCR)-based technique than others such as random amplified polymorphic DNA (RAPD), because it produces many more polymorphic fragments. The sequence-related amplified polymorphism (SRAP) technique, which has been applied to gene tagging in *Brassica* plants, is a relatively new and highly efficient PCR-based technique (Li and Quiros 2001). It is based on PCR amplification using two primers and can produce highly reproducible polymorphic bands similar to the AFLP technique (Li and Quiros 2001; Vos et al. 1995). However, the SRAP technique is simpler and less costly than AFLP because it omits the enzyme restriction, ligation of primer adapters, and pre-amplification in the AFLP technique. Although genetic diversity studies have been conducted on *Apiosporina morbosa* and *Gibberella zeae* pathogens using the SRAP technique (Fernando et al. 2006; Zhang et al. 2005), no information has been reported on genetic variation and population differentiation of *S. sclerotiorum* using this technique. To obtain information on genetic structure of the *S. sclerotiorum* populations in sunflower, the SRAP technique was used to develop molecular markers. The objectives of this research were to (1) determine the genetic variation and structure of the *S. sclerotiorum* population in sunflower using SRAP analysis, and (2) analyze the natural selection pressures causing genetic diversity and restriction of gene flow among populations.

Materials and methods

Isolate collections To analyze genetic diversity and differentiation of *S. sclerotiorum* populations, 96 isolates of the pathogen were used in the study. The isolates from IM in China were divided into three populations: the West IM, Middle IM and East IM sets, according to their geographic origins and local

sunflower growing layout. The West IM set included 30 isolates from five counties, six isolates sampled from three fields in each county; the Middle IM set included 25 isolates from five counties, five isolates sampled in each county; and the East IM set included 16 isolates from four counties, four isolates sampled in each county. The Canadian population included 21 isolates sampled from seven fields, each field with three isolates; and the UK population included four isolates from four counties. All isolates were obtained from sclerotia. Sclerotia were surface-sterilized using 10% commercial bleach (0.5% NaClO) for 3 min, washed three times with sterile water, then placed on potato dextrose agar (PDA) and incubated for 72 h at room temperature (20–24°C). The mycelial tips of each isolate were cut and re-cultured three times to obtain the genetically identical cultures for DNA extraction.

DNA extraction A mycelial disc from a 3-day-old culture was inoculated on a PDA plate. After incubation for 4–7 days, mycelia were harvested by toothpick and transferred into a 2-ml centrifuge tube. Mycelia were dried 12 h in a Freeze Dryer (LABCONCO) at −100°C, 0–5 μHg vacuum and stored at −80°C. DNA of each isolate was extracted following the Fernando lab protocol: The mycelia were put into a 1.5 ml centrifuge tube and frozen in liquid nitrogen. Mycelia were ground in 600 μl TES buffer, to which was added 140 μl 5 M NaCI and 65°C 70 μl 10% CTAB, then vortexed and incubated at 65°C for 30 min. To the tube, 600 μl chloroform: isoamyl alcohol (24:1) was added, mixed for 1 min, then centrifuged for 15 min at 10,000 rpm, and the aqueous layer was transferred to a clean centrifuge tube. This step was repeated, then 80 μl 5 M NaCI and 1,000 μl 100% ethanol was added and allowed to stand for 5 min to precipitate DNA, before centrifuging for 5 min at 13,000 rpm. The supernatant was poured off and the pellet washed with 200 μl chilled (−20°C) 80% ethanol. The pellet was dried 20–30 min upside down in a fume-hood and resuspended in 400 μl warm (65°C) sterile distilled (sd) H_2O before adding 2.7 μl RNAse and Proteinase K to 400 μl DNA solution. The mixture was allowed to stand for 10 min and incubated at 37°C for 30 min. Then 400 μl chloroform was added and the above steps repeated, the pellet was resuspended in 100 μl warm (65°C) sdH_2O. All DNA extracts were quantified using a spectrophotometer and adjusted to a final concentration of 5 ng μl^{-1} for PCR analysis.

Amplification of SRAPs The PCR-based SRAP technique was used to analyze genetic diversity and population differentiation in the fungi according to Zhang et al. (2005). PCR amplification reaction with the SRAP primers was performed in a 15-μl reaction volume containing 15 ng template DNA, 0.4 μM each of two primers, 0.75 unit of Taq polymerase (Invitrogen Life Technologies, ON, Canada), 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM each of dNTPs. The fragments were amplified in a programmable thermal controller (PTC-200, MJ Research, Inc., Waltham, MA, USA). The first five cycles were run at 94°C for 1 min, 35°C for 50 s, and 72°C for 1 min, for denaturing, annealing and extension, respectively. The remainder of the amplification consisted of 35 cycles at 94°C for 50 s, 50°C for 5 s, and 72°C for 1 min. PCR repetitions using the same set of primers and isolates and different DNA preparations of the same isolates were conducted to check the reproducibility of results. To select effective primer combinations, 65 pairs of the SRAP primers were screened for the PCR amplification against 32 isolates representing populations from different regions. All the PCR products were separated in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems and Hitachi, Ltd., Japan) to examine polymorphic bands.

Based on efficacy of primers producing polymorphic bands, out of 65 primer combinations, seven pairs of primers were chosen for generating the SRAP data: FC1+ODD4, FC1+BG56, SA7+ODD4, SA7+BG39, BG23+BG66, BG23+68, and ODD20+BG92 (Table 1). The final examination of polymorphism and fragment analysis was done using an ABI 3100 Genetic Analyzer. The protocols of the manufacturer were followed to fill polymer gel fluid, load PCR products, set the preferences of the data collection software, monitor the analysis run, and analyze raw data. Amplified fragments between 100 and 500 bp in size were scored by using the software Genographer (version 1.6, Montana State University) and adjusted manually as putative loci with two alleles, one allele indicating the presence of a fragment (designated as 1) and the other the absence (designated as 0) of homologous bands to create a binary matrix of isolates and molecular fragments of the different SRAP phenotypes. Bands >500 or <100 bp in size were not scored because the resolution was insufficient to discriminate between bands.

Statistical analysis Populations were defined according to their geographic locations. The software Population Genetic Analysis (POPGENE, version 1.32; Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada), and Tools for Population Genetic Analysis (TFPGA, version 1.3; Northern Arizona University, Flagstaff, AZ, USA) were used for statistical analysis of standard population genetics. The mean genetic diversity, H, was calculated as $H=(1-\Sigma Pi^2)$, where Pi is the frequency of allele i at the locus (Nei 1973). Heterozygosity and percent polymorphic loci (99% criterion) were estimated for all populations. Genotypic diversity was calculated by Shannon's information index used to measure biodiversity in categorical data. The index takes into account the number of species and the evenness of the species. It is increased either by having additional unique species, or by having greater species evenness (Shannon and Weaver 1949). Differentiation among populations was estimated using an exact test (Raymond and Rousset 1995) and by indirect estimation of gene flow using Gst with $Nm=1/4(1-Gst)/Gst$ (Nei 1973; Slatkin 1987), where N is the effective population size, m is the immigration rate of gene flow, and Nm is the average number of migrants among populations per generation (Slatkin 1987; Slatkin and Barton 1989). Cluster analysis of multilocus SRAP genotypes was based on allele frequencies observed for each population. A phenogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA) from a Nei's genetic distance matrix (Slatkin 1987) using the TFPGA 1.3 software package. Bootstrap sampling (1,000 replicates) was performed for statistical support of branches of the constructed phenogram (Felsenstein 1985). The analysis of molecular variance (AMOVA) was used to partition the total genetic variance within and among populations from different geographic regions (Excoffier et al. 1992). The AMOVA was performed by treating a SRAP profile as a haplotype, using Arlequin software (version 2.0) provided by L. Excoffier (Dept. of Anthropology and Ecology, University of Geneva, Switzerland).

Results

Variations within populations Based on efficacy of primers producing polymorphic bands, seven pairs of SRAP primers (Table 1) out of 65 primer combinations were chosen for generating the SRAP data and 123 polymorphic loci were obtained. Populations of *S. sclerotiorum* isolated from the five geographic regions showed various levels of genetic variability (Table 2, Fig. 1). The number (pl) and percent (r, based on the 99% criterion) of polymorphic loci varied from 38 and 30.89%, respectively, in the population collected from the UK to 120 and 97.56%, respectively, in the Middle IM population from China. The values of Shannon index (i) revealed that there were high genotypic diversities in the five populations, which varied from 0.1876 in the UK population to 0.5301 in the West IM population. The heterozygosity (h) of the five geographic populations obtained by estimating allele frequency based on a Taylor expansion varied from 12.91% in the UK population to 35.44% in the West IM population.

Population differentiation Genetic identity and distances differed among five geographic populations (Table 3). Genetic identity, as presented by Nei's unbiased identity index (Nei 1973), was high and similar among geographic populations. These values ranged from 0.6477 between the populations of West IM and the UK to 0.974 between the populations from East IM and Canada. The UPGMA cluster analysis using Nei's genetic distance (Nei 1973) gave distances ranging from 0.0259 to 0.4343 for geographic populations (Table 3). The four geographic populations were clustered into one subgroup and were distinctly differentiated from the UK population (Fig. 2). It seems that the genetic distance is positively related to the geographic origins in populations from

Table 1 Primers for generating the sequence-related amplified polymorphism data

Primer	Sequence
FC1	5′-TCAAGGGCAGGTAAGAACAA-3′
SA7	5′-CGCAAGACCCACCACAA-3′
BG23	5′-ATTCAAGGAGAGTGCGTGG-3′
ODD20	5′-TCGTTGTTATGGCTGGAGA-3′
ODD4	5′-AGG GTA GCG TCT GAG GA-3′
BG39	5′-AACAACAAAGACTACCTCCC-3′
BG92	5′-AGGTGAGTAAGTTCGGACAT-3′
BG66	5′-GATTTTGATTTACAGGAGAGA-3′
BG56	5′-GAGAAAGGTATGAGTTGAAC-3′
BG68	5′-AAAGGGAGACAGATATTTACA-3′

Table 2 Genetic diversity among five *Sclerotinia sclerotiorum* populations based on sequence-related amplified polymorphism fingerprinting

Population	n	g	na	ne	h	I	pl	*r* (%)
West Inner Mongolia (IM)	30	30	1.9675	1.5894	0.3544	0.5301	119	96.75
Middle IM	25	25	1.9756	1.5829	0.3434	0.5140	120	97.56
East IM	16	16	1.9675	1.5414	0.3267	0.4952	119	96.75
Canada	21	21	1.9512	1.5792	0.3352	0.5005	117	95.12
UK	4	4	1.3089	1.2276	0.1291	0.1876	38	30.89

n population size, *g* number of genotypes in populations, *na* observed number of alleles, *ne* effective number of alleles, *h* Nei's gene diversity (1973), *I* Shannon's Information index, *pl* number of polymorphic loci, *r (%)* percentage of polymorphic loci

IM. The populations with nearer geographic origins were more closely clustered genetically. However, the populations from East IM and Canada as well as those from West IM and Middle IM were more closely related to each other than to the UK population.

The population differentiation of *S. sclerotiorum* was expressed by probability of pairwise comparison using the exact test. Results indicated that significant differentiation was observed between populations from different regions ($P<0.05$; Table 4). In general, the populations clustered in the same lineage were not differentiated from each other (Table 4, Fig. 2). The estimation of gene flow between populations using *Nm* as a measurement of population differentiation showed the rates of gene flow ranged from 1.5406 between the West IM and UK populations to 18.4149 between the West IM and Middle IM populations (Table 4). The highest gene flow was observed between the populations from West IM and Middle IM ($Nm=18.4149$, $P=0.4615$) and between those from East IM and Canada ($Nm=17.5986$, $P=1.0000$). These pairs of populations were not significantly differentiated from each other based on the probability of pairwise comparisons. The largest population differentiation occurred between the West IM and UK populations ($Nm=1.5406$, $P=0.0000$; Table 4, Fig. 2).

The results of AMOVA analysis indicated that 94% of the total molecular variance was due to differences within geographic regions and only 6% of the variance was associated with differences among regions. Both the variances within and among geographic regions were significant at $P=0.001$ (Table 5).

Discussion

In the current study, the four populations from West IM, Middle IM, East IM and Canada were clustered into one subgroup in which the isolates from West and Middle IM belonged to one population, while those from East

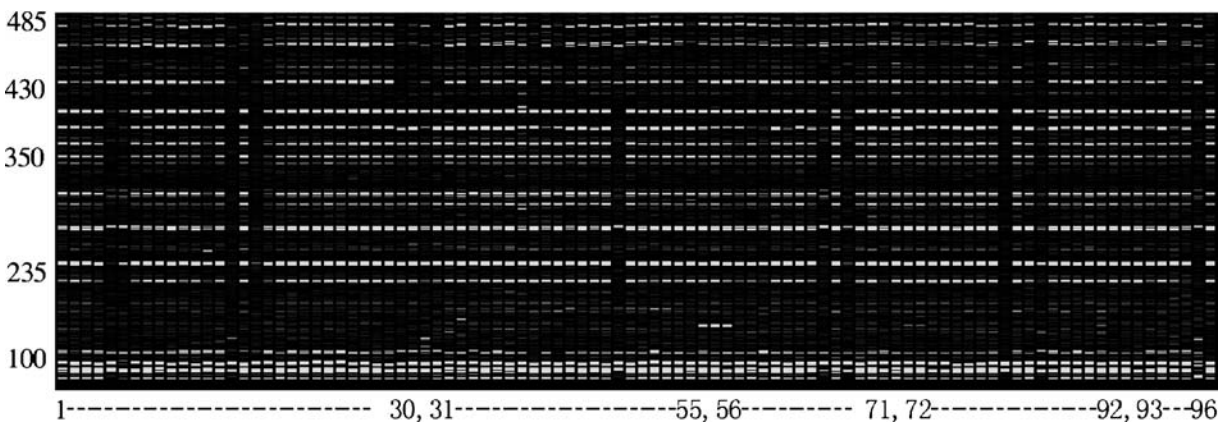

Fig. 1 A gel separating the polymerase chain reaction products amplified with the sequence-related amplified polymorphism primers SA7 and BG39 against DNA samples of *Sclerotinia sclerotiorum* isolates. The gel was reconstructed by the Genographer program using the fragment analysis data generated by the ABI Prism 3100 Genetic Analyzer, and showed genetic diversity among the five populations: West Inner Mongolia (*IM*) (*lanes 1–30*), Middle IM (*lanes 31–55*), East IM (*lanes 56–71*), Canada (*lanes 72–92*) and UK (*lanes 93–96*)

Table 3 Pairwise comparison of the genetic diversity and distance among five geographic populations of *Sclerotinia sclerotiorum*

Population	West IM	Middle IM	East IM	Canada	UK
West Inner Mongolia (IM)	–	0.974	0.9488	0.9609	0.6477
Middle IM	0.0263	–	0.9456	0.9428	0.6876
East IM	0.0525	0.0559	–	0.9744	0.8043
Canada	0.0398	0.0589	0.0259	–	0.7718
UK	0.4343	0.3745	0.2177	0.259	–

Nei's genetic identity based on 123 sequence-related amplified polymorphism loci is above the diagonal, and Nei's genetic distance coefficients are below the diagonal

IM and Canada essentially were another population without differentiation. The isolates from UK formed a population that was significantly differentiated from other populations. This is the first report indicating that there are two genetic populations of *S. sclerotiorum* in sunflower in Inner Mongolia Autonomous Region, China. Sun et al. (2005) compared *S. sclerotiorum* populations in oilseed rape from China, Canada, Poland and Slovakia with RAPD and found highly significant genetic variation among and within populations, with the five populations divided into three distinct clusters. In 2006, Yu et al. (2006) used RAPD to analyze the polymorphism of *S. sclerotiorum* isolates from sunflower in China. Although a small set of isolates were investigated, they were divided into five groups. Mert-Türk et al. (2007) assessed genetic variation of *S. sclerotiorum* isolates in Turkey by microsatellite and morphological markers and found a high genotypic diversity level of 63% within the fungal population. The results in this study agree with the above findings that there exists a high level of genetic diversity within and among the *S. sclerotiorum* populations.

The results showed that the West IM and Middle IM populations are closely clustered and have the highest gene flow (Nm=18.4149, P=0.4615), suggesting that essentially neither population had significant differentiation. This is not surprising. West IM and Middle IM are two major sunflower production areas in IM. Although the two areas are approximately 300 km apart, sunflower fields are actually contiguous between two regions, providing a pathway of cross-infection for pathogen isolates from the two areas. In addition, both production areas share the same growth season, similar weather conditions, soil structures and agricultural practices, suggesting that no barrier exists in ecological adaptation of the pathogen isolates in either of these areas. Frequent seed exchanges have been occurring and the same varieties have been planted in both areas for many years, indicating that the host selection pressures are identical in both pathogen populations. All of these factors might result in no differentiation between two pathogen populations. However, both populations have the highest heterozygosity (35.44%) among the five populations. This might be caused by diversity of sunflower varieties in the two areas. Diversity of host genotypes from which pathogens were isolated has been pointed out as one of the factors responsible for the high value of genetic diversity of pathogens found in other host and pathogen systems (González et al. 1998; Sun et al. 2005; Zhang et al. 2005). Since 1990, there have been more than 100 sunflower cultivars imported from North America, Australia and Canada in both regions. The genotypic diversity of sunflower cultivars probably has led to the evolution within the populations, and thus the high genetic diversity of *S. sclerotiorum* populations in two regions.

Sun et al. (2005) indicated that the isolates from the Qinghai and Anhui provinces of China were significantly different according to the genetic identity, genetic distance, and genetic diversity. In the present

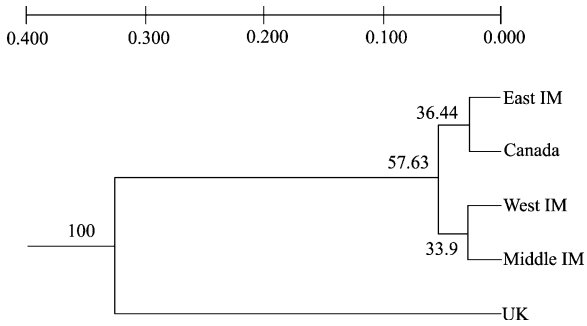

Fig. 2 Phenogram of Rogers' modified genetic distance between *Sclerotinia sclerotiorum* populations isolated from five regions. *Numbers at branches* indicate the percentage of occurrence of the cluster in 1,000 bootstrapped phenograms. West IM (Inner Mongolia) population; Middle IM population; East IM population; Canada population; and UK population

Table 4 Pairwise comparison of gene flow and probability of population among five geographic populations of *Sclerotinia sclerotiorum*

Population	West IM	Middle IM	East IM	Canada	UK
West Inner Mongolia (IM)	–	18.4149	6.5684	11.2163	1.5406
Middle IM	0.4615	–	6.6367	8.8998	1.6475
East IM	0.0000	0.0000	–	17.5986	3.8664
Canada	0.0001	0.0000	1.0000	–	3.2306
UK	0.0000	0.0179	1.0000	1.0000	–

Estimates of the number of migrants (Nm) between populations are above the diagonal; probabilities of each pairwise comparison using the exact test are below the diagonal.

study, the population from East IM is significantly differentiated from populations in West and Middle IM, whereas the UK population formed a unique lineage differentiated from others. The population differentiation may result from the reproductive isolation due to geographical separation. An early report (Bowden and Leslie 1999) showed that sexual reproductive isolation is considered as an important assumption for molecular divergence among populations from different geographic regions. The longer the period of reproduction isolation, the greater the divergence in genotypes is, because gene flow has been restricted between populations from isolated regions (Bowden and Leslie 1999). This assumption might explain population differentiation between West and Middle IM populations on the one hand and East IM populations on the other, or between the UK and other populations. Unlike West and Middle IM, East IM is a remote sunflower production area, more than 2500 km away from Middle IM. There is little sunflower cultivation between East IM and Middle IM sunflower production areas, preventing spread between West IM or Middle IM and East IM populations. Moreover, there is no frequent exchange of sunflower seeds and products between West IM and Middle IM production areas on the one hand, and East IM production areas on the other. Because the weather in East IM is colder than that in West and Middle IM, the flowering period of sunflower is delayed in the season in East IM. Different flowering periods of sunflower between West and East IM avoided the optimum time of infection if there might be *S. sclerotiorum* isolates transmitted from West to East IM. These all may contribute to reproductive isolation between two populations and lead to the independent genetic evolution and thus differentiation between the two populations.

Sun et al. (2005) reported that the relationship between Canadian and Polish populations of *S. sclerotiorum* was very close, whereas the geographic distance between them was large. The results showed that isolates from East IM in China and Manitoba in Canada were clustered in the same lineage without differentiation. We do not know why a close similarity exists between the two populations. Both East IM and Manitoba, Canada, are at a high latitude North (over 52°), and have similar climates. Whether a similar ecotype in two sunflower production areas might produce genetically similar populations requires further investigation.

Analysis of the diversity and differentiation of *S. sclerotiorum* populations showed that epidemics of sunflower stem and head rots in IM are caused by genetically diverse populations of *S. sclerotiorum*. Regardless of geographic location, the major source of genetic variation came from differences among

Table 5 Analysis of molecular variance for populations from five geographic populations of *Sclerotinia sclerotiorum* using sequence-related amplified polymorphism markers

Source of variation	*df*	Sum of squares	Variance components	*P* value	Percentage of variation
Among populations	4.00	176.32	1.29791Va	<0.001	5.96
Within populations	91.00	1,864.92	20.49366Vb	<0.001	94.04
Total	95.00	2,041.24	21.79		

Fixation Index (FST), 0.05956

isolates within populations. These findings have implications on the disease management. Resistant cultivars are one of the most effective means of disease control. Because of high levels of genetic diversity within populations, the expression of disease resistance to *S. sclerotiorum* may be dependent on the strain of the pathogen. When screening breeding lines for resistance to *S. sclerotiorum* in sunflower, it is advisable that several strains, or a broad selection of isolates, should be used to effectively screen sunflower germplasm for resistance in a region. Based on population differentiation, we also suggest that representative strains from East IM and West IM should be used for inoculating hybrids or lines obtained from breeding programs to find *S. sclerotiorum* resistance corresponding to different geographic regions.

More research is needed before firmer conclusions can be made with respect to selection pressures for genetic diversity and population differentiation of *S. sclerotiorum*; nevertheless, the results of the present study serve as a baseline of information for monitoring population evolution of *S. sclerotiorum* in Inner Mongolia, China, in the future.

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