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Distribution of mating-type alleles and genetic variability in field populations of *Leptosphaeria maculans* in western Canada

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Abstract

Leptosphaeria maculans is the most important fungal pathogen of canola (*Brassica napus*, oilseed rape) that causes the devastating stem canker in canola fields of western Canada. The population genetic structure of *L. maculans*, represented by nine subpopulations from a 6-year period and three different provinces in western Canada, was determined using ten minisatellite markers. Isolates collected at different locations in six consecutive years had an even distribution of *MAT1-1* and *MAT1-2* across the nine subpopulations. All subpopulations of *L. maculans* exhibited a moderate gene diversity (*H* = 0.356–0.585). The majority of the genetic variation occurred within subpopulations. Approximately 8% and 4% of the variations were distributed between sampling year and location, respectively. Genetic distance (F_{ST}) results, using analysis of molecular variation (AMOVA), indicated that subpopulation pairing within isolates by year ranged from F_{ST} = 0.010 to 0.109, and the location subpopulation ranged from F_{ST} = 0.038 to 0.085. Bayesian clustering analyses of multiloci inferred two distinct clusters in all the subpopulations examined. This study indicates a relatively high degree of gene exchange between the different *L. maculans* isolates. Our results suggest that this can occur in the wide growing areas of canola fields in western Canada. This gene exchange produced different gene allele frequencies and divergence between populations.

KEYWORDS
blackleg, *Brassica napus*, field population, *Leptosphaeria maculans*

1 | INTRODUCTION

Blackleg, also known as Phoma stem canker, is caused by *Leptosphaeria maculans*. It is an important disease in canola (*Brassica napus*) and other cruciferous crops in Europe, Australia and Canada (Balesdent, Louvard, Pinochet, & Rouxel, 2006). In Canada, canola is the most important cash crop with a production of approximately 13.5 million tonnes in 2015 (Fernando, Zhang, & Amarasinghe, 2016). Blackleg disease has been reported to result in great yield losses in canola production in western Canada. Gugel and Petrie (1992) reported seed yield loss of up to 50% in severely infected fields in Saskatchewan. Since then, extensive reports revealed canola yield losses of 10%–50% due to blackleg disease (Barbetti & Khangura, 1999; Chen & Fernando, 2006; Hall, Peters, & Assabgui,1993; Hwang et al., 2016; Zhou et al., 1999). Blackleg disease severity and extent of yield loss in canola production depend on climate, geographic distribution and cultivars grown. In the last two decades, blackleg has been mainly managed by crop rotation and utilization of resistant cultivars (Fernando et al., 2016).

A variety of genetic markers have been used to examine diversity and genetic variability of the field population of *L. maculans* isolates. Techniques, such as AFLP (amplified fragment length polymorphism), RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA), were first used to investigate the genetic diversity of oilseed rape field populations globally (Barrins, Purwantara, Ades, Salisbury, & Howlett,

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2002; Goodwin & Annis, 1991; Jedryczka, Irzykowski, Jajor, & Korbas, 2010; Pongam, Osborn, & Williams, 1999; Purwantara, Barrins, Cozijnsen, Ades, & Howlett, 2000). Subsequent to these techniques, newer methods based on variable number tandem repeat loci, such as minisatellite and microsatellites, have been widely adopted as they produce size-specific alleles based on the repeat number. Minisatellites are 10–60 bp series of short repeats and microsatellites with 1–6 bp repeated motif. These are popularly used as molecular markers for genetic distance and population diversity studies. A number of minisatellites from *L. maculans* have been identified and characterized in the last two decades (Attard et al., 2001; Eckert et al., 2005; Jedryczka et al., 2010). Genetic structure of *L. maculans* populations has been analysed using minisatellites and microsatellites in Australia (Hayden, Cozijnsen, & Howlett, 2007; Hayden, Wilson, Cozijnsen, & Howlett, 2004), France (Gout et al., 2006) and Mexico (Dilmaghani et al., 2012). Dilmaghani et al. (2012) analysed migration patterns and changes using 14 minisatellite markers in the worldwide spread of *L. maculans* population, including some isolates from western Canada.

Like other ascomycetes, *L. maculans* has a single mating type (*MAT1-1* or *MAT1-2*) with two idiomorphs. Isolates must carry different alternate forms (idiomorphs) to be able to mate (Cozijnsen & Howlett, 2003). Sexual reproduction by the initial production of ascospores is important to generate genetic variation in *L. maculans* population. Very few studies have examined the mating-type locus frequency and genetic variation of the *L. maculans* population in western Canada. In this study, a large number of *L. maculans* isolates collected from the western Canadian provinces of Alberta, Saskatchewan and Manitoba in six consecutive years (2010–2015) were used for analysis. The mating-type alleles, population structures and genetic diversity of *L. maculans* isolates from western Canada were investigated.

2 | MATERIALS AND METHODS

2.1 | Isolate collection

A total of 602 *L. maculans* isolates were obtained from pycnidiospores oozing from pycnidia isolated from infected canola stubble. The isolates collected were as follows: 96 isolates collected in 2010 (32 isolates from each of Manitoba, Alberta and Saskatchewan), 96 isolates in 2011(32 isolates from each of Manitoba, Alberta and Saskatchewan), 96 isolates in 2012 (94 and 2 isolates from Manitoba, and Saskatchewan, respectively), 124 isolates in 2013 (120 and 4 isolates from Manitoba, and Saskatchewan, respectively), 94 and 96 isolates from Manitoba in 2014 and 2015. In total, 64, 468 and 70 isolates were collected from Alberta, Manitoba and Saskatchewan, respectively. All isolates were cultured and maintained on V8 juice agar plates amended with 0.35% (v/w) streptomycin sulphate. Pycnidiospores and mycelia were harvested by flooding the cultures with 2 ml distilled water, and used for DNA extraction (Liban, Cross, Kutcher, Peng, & Fernando, 2016).

2.2 | DNA extraction

Genomic DNA was extracted from a mixture of pycnidiospores and mycelia using a modified CTAB method, as described by Liban et al. (2016). Briefly, 2× CTAB buffer was added to a sample, homogenized using 0.2 mm ceramic beads for 45 s at 4,724 *g* in a Precellys® 24 homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France), then incubated at 65°C for 20–30 min. Extraction was performed using phenol/chloroform/isoamyl (25:24:1), precipitated with 95% ethanol (v/v) and 150 μl 5 M NaCl, and then washed twice with 500 μl 70% ethanol. Finally, the DNA pellet was dissolved in 100 μl DNase-free water.

2.3 | Mating-type allele determination and minisatellite analysis

A multiplex PCR assay (Cozijnsen & Howlett, 2003) was employed to determine the mating-type idiomorphs of *L. maculans* isolates (Table 1). The *MAT1-1* and *MAT1-2* isolates were identified with a single band of 686 and 443 bp, respectively. The distribution of the two different mating-type alleles within the 602 isolates in each subpopulation was analysed using a Chi-squared test.

A total of 10 polymorphic minisatellite loci in *L. maculans* were used to examine the genetic diversity of the 602 isolates (Table 1). PCR amplification was performed in 25 μl total volume of reaction mixture containing: 10–50 ng DNA, 25 pmol of each primer, 3.75 mM $MgCl_2$, 1.25 mM of each deoxynucleotide triphosphate and 1.25U EconoTaq DNA polymerase (Econo Taq DNA polymerase; Lucigen, Middleton, WI, USA) with 2.5 μl supplied buffer. The PCR thermocycling steps were as follows: 95°C for 3 min of denaturation; 30 cycles at 95°C for 15 s, annealing for 30 s and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were separated and visualized by electrophoresis on a 2.0% agarose gel stained with Redsafe (iNtRON Biotechnology, Seongnam-Si, Gyeonggi-do, Korea). Each allele identified in the ten minisatellite loci was sequenced to determine the exact allele size and repeat motif. The genotype data were scored based on the allele identified at each minisatellite locus in each sample.

2.4 | Data analysis

For statistical analysis, the 602 isolates collected were divided into nine subpopulations: (i) according to year: pop10 (2010), pop11 (2011), pop12 (2012), pop13 (2013), pop14 (2014) and pop15 (2015) and (ii) location: popAB (Alberta), popMB (Manitoba), and popSK (Saskatchewan). Genetic diversity was analysed by examining the genotype and gene diversity of each subpopulation. For each population, POPGENE software V.1.32 was employed to calculate the allele frequency and Nei's gene diversity (*h*) (Yeh, Yang, Boyle, Ye, & Mao, 1997). The average allele, also termed mean gene diversity (*H*) across each locus within each subpopulation, was estimated. Genetic structure was computed with Nei's

TABLE 1 List of primer pairs for minisatellites markers and mating types examined in this study

Primer name	Sequence (5′–3′)	Annealing temperature (°C)	Reference
MinLm4F	ACCAGGTGGAGTTGATAACAT	55	Eckert et al. (2005)
MinLm4R	TCCTGCGAATCCCATTAG		
MinLm5F	TACGCTTCTCATTCGGTCCT	60	Eckert et al. (2005)
MinLm5R	CGGGTGGCAGCATTCAC		
MinLm6F	GGAAGGAACACACGGTGAC	60	Eckert et al. (2005)
MinLm6R	AATTGAATGATTTGCGACACA		
MinLm555F	CACTGTCATTCCTCCTCCTGGTT	60	Jedryczka et al. (2010)
MinLm555R	GCAGCCGTTTAGTTCTCCATTT		
MinLm585F	GTCCAAGAGGGGTCTAATG	60	Jedryczka et al. (2010)
MinLm585R	TGCAATACCTATCAACTATGCTA		
MinLm935-2F	AGTAGGCAACACAACAGCACACA	58	Jedryczka et al. (2010)
MinLm935-2R	CCCTCTCTGCCATTTTCCATTAG		
MinLm1139F	ACGACGCGGAAGGGTTTT	54	Jedryczka et al. (2010)
MinLm1139R	ACCATCTACCTCATGCCCTGAAC		
MinLm2448F	TTGAGCCTACTTGGGGAACA	60	Jedryczka et al. (2010)
MinLm2448R	AAGTGGCTAGTGGATTGGAAGAT		
MinLm2451F	GGGGCGAATGGTATGTTTATAGT	58	Jedryczka et al. (2010)
MinLm2451R	CGGACACAATACTCACCACCTC		
MinLm2452F	GTACATGGGCGGACAGGC	60	Jedryczka et al. (2010)
MinLm2452R	CATTTACACTGCACACCTGCTCA		
MAT1-1-F	CTCGATGCAATGTACTTGG	58	Cozijnsen and Howlett (2003)
MAT1-2-F	AGCCGGCGGTGAAGTTGAAGCCG		
MAT-R	TGGCGAATTAAGGGATTGCTG		

coefficient of population differentiation (G_{ST}) using the GENEPOP program (Raymond & Rousset, 1995) to indicate the proportion of genetic variation that contributed to population differentiation. A hierarchical analysis of molecular variation (AMOVA) was carried out using Arlequin version 3.5 program (Excoffier & Lischer, 2010) to estimate the partition of genetic variability within the subpopulation.

Population structure was implemented independently in the program STRUCTURE version 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) with a Bayesian clustering approach to assign individuals to a "genetic" population using the minisatellite genotype data. Different potential values of the number of populations (*K*) were computed with independent allele frequency for all isolates under the admixture model. Then, 10 iterations running were performed for each *K* value, ranging from 1 to 10 with 50,000 burn-in replicates, followed by 10^5 MCMC (Markov chain Monte Carlo) simulations. The optimal *K* value was calculated as described by Pritchard et al. (2000) and Evanno, Regnaut, and Goudet (2005). The results were interpreted and harvested by STRUCTURE HARVEST (Earl & vonHoldt, 2012) after processing all runs. The final outputs from the 10 replicates were averaged using CLUMPP (Jakobsson & Rosenberg, 2007).

3 | RESULTS

3.1 | Mating-type distribution

Multiplex PCR products corresponding to one of the two different mating-type alleles were obtained in all the analysed 602 *L. maculans* isolates. Both mating types (*MAT1-1* and *MAT1-2*) were present in the different subpopulations regardless of the year and/or location that isolates were collected from. A total of 300 isolates were found to be *MAT1-1* and 302 isolates were *MAT1-2*, indicating an equal ratio of 49.8:50.2%, respectively (Table 2). There was no significant deviation from the 1:1 ratio between the two mating types in the investigation of all the isolates ($p = .935$). The two mating-type idiomorphs were distributed equally and retained the 1:1 ratio in each subpopulation (Table 2). No significant deviation of mating-type alleles was detected in the population— total, year and location— suggesting that there was no restriction in the sexual recombination in *L. maculans* populations in western Canada.

3.2 | Minisatellite variation

The 10 minisatellite loci tested in this study were polymorphic in the populations. A total of 31 alleles were detected in all the examined

TABLE 2 Distribution of the mating-type idiomorphs of *Leptosphaeria maculans* in populations of western Canada

Group by year and geographic origin	Subpopulation	*n*	Number of alleles for mating type		χ^2	*p* value
			MAT1-1	*MAT1-2*		
Year scale						
2010	pop10	96	48	48	0.000	1.000
2011	pop11	96	46	50	0.167	.683
2012	pop12	96	42	54	1.500	.221
2013	pop13	124	62	62	0.000	1.000
2014	pop14	94	48	46	0.043	.837
2015	pop15	96	53	43	1.042	.307
Location scale						
Alberta	popAB	64	35	29	0.563	.453
Manitoba	popMB	468	231	237	0.077	.782
Saskatchewan	popSK	70	34	36	0.571	.811
Total		602	300	302	0.007	.935

minisatellite loci in 602 isolates. In detail, at locus MinLm4, only one allele was detected in subpopulations pop11, pop12, pop14, pop15 and popAB and the rest of the subpopulations had the two allele types with a different number of repeated motifs. The other nine minisatellites (MinLm5, MinLm6, MinLm555, MinLm585, MinLm935-2, MinLm1139, MinLm2448, MinLm2451 and MinLm2452) demonstrated a high level of allele variation in all subpopulations, with 3, 2, 2, 6, 4, 4, 2, 3 and 3 different alleles detected, respectively (Table 3). The length of repeat motif of each allele sequenced in this study ranged from 17 to 90 bp, which agrees with previous reports (Eckert et al., 2005; Jedryczka et al., 2010). Interestingly, all the alleles were found to be present in popMB. This is probably due to the large population size of isolates collected in Manitoba, comprising 468 isolates (Table 3).

3.3 | Genetic diversity within populations

The most frequent allele of 2× at locus MinLm585 was detected only in Manitoba in 2012, and 6× at locus MinLm5 was present in Manitoba in 2013–2015, (Table 3). MinLm2451 and MinLm2452 both had three different alleles, with 3× allele being the most frequent at 0.500–0.943 and 0.863–1.000, respectively, in all nine subpopulations. The most frequent allele, at loci MinLm585, MinLm935-2, and MinLm2448, was consistent across the nine subpopulations (Table 3). However, locus MinLm555 had the most frequent allele, with 5× being in subpopulations of popAB and pop11 at 0.677 and 0.563, respectively, while in all other subpopulations, 4× was the most frequent allele. The different allele distributions in subpopulations of popAB and popMB at locus MinLm555 and MinLm1139 may have been affected by the adequate sample sizes needed in the investigation. Gene diversity ranging from $H = 0.356–0.585$ was moderate to high for the nine subpopulations. The mean gene diversities at each locus (h) ranged from 0.025 to 0.681 and from 0.020 to 0.680 for the six subpopulations based on year and three subpopulations based on location, respectively (Table 3).

Excluding MinLm4, MinLm2448 and MinLm2452, significant genetic differentiation at the other seven loci ($F_{st} = 0.041–0.154$) was observed over the 6-year period (Table S1). However, there was no significant genetic differentiation for the 10 minisatellites across the subpopulations from the three different locations (Table S1). These results also reflect the low minisatellite variations of MinLm4, MinLm2448 and MinLm2452.

3.4 | Genetic differentiation between populations

Hierarchical AMOVA indicated that 8% and 92% of the variations were attributed to between and within subpopulations by year. The same situation was observed in the location subpopulations where at least 4% and 96% of the diversity occurred from between and within the populations, respectively. To summarize, the population of popMB showed the greatest variation at 95.3% within population. This genetic differentiation was consistent with the genetic distance analyses (F_{ST}), where moderate levels of genetic differentiation were found between different years and between different locations, ranging from 0.010 to 0.109 and 0.038 to 0.085, respectively. In the other words, the genetic identity (*NeI*) values were ranged from 0.891 to 0.990 and 0.915 to 0.975 between year subpopulations and between location subpopulations, respectively (Table 4). There were no significant genetic distinctions between each year subpopulation or between each location subpopulation through pairwise comparison analysis. The highest genetic distance value (F_{ST} = 0.109, 0.106, 0.053, 0.053, and 0.069) was always estimated between subpopulation pairs of pop13 and the other years' populations (pop10, pop11, pop12, pop14 and pop15). The highest genetic distance value (F_{ST} = 0.085, *NeI* = 0.915) was detected between the subpopulations of *L. maculans* isolates collected from Manitoba and Saskatchewan. Based on these findings, we conclude that the

TABLE 3 Allele frequencies, gene diversity of the nine subpopulations of *Leptosphaeria maculans* at ten minisatellite loci

Locus	Allele[a]	Repeat Motif (bp)	Allele frequency of year scale population						Gene diversity (h)[c]	Allele frequency of location scale population			Gene diversity (h)[c]
			pop10 96[b]	pop11 96	pop12 96	pop13 124	pop14 94	pop15 96		popAB 64	popMB 468	popSK 70	
MinLm4	3×	51	0.990	1.000	1.000	0.935	1.000	1.000	0.025	1.000	0.984	0.986	0.020
	2×		0.010	0.000	0.000	0.065	0.000	0.000		0.000	0.016	0.014	
MinLm5	4×	16	0.854	0.885	0.750	0.347	0.755	0.677	0.428	0.781	0.660	0.900	0.348
	3×		0.146	0.115	0.250	0.629	0.234	0.146		0.219	0.298	0.100	
	6×		0.000	0.000	0.000	0.024	0.011	0.177		0.000	*0.042*	0.000	
MinLm6	2×	21	0.958	0.917	0.927	0.806	0.723	0.896	0.224	0.906	0.854	0.957	0.171
	4×		0.042	0.083	0.073	0.194	0.277	0.104		0.094	0.146	0.043	
MinLm555	4×	63	0.469	0.323	0.594	0.629	0.564	0.479	0.500	0.438	0.520	0.514	0.500
	5×		0.531	0.677	0.406	0.371	0.436	0.521		0.563	0.480	0.486	
MinLm585	4×	26	0.063	0.010	0.063	0.089	0.106	0.052	0.631	0.063	0.074	0.000	0.579
	5×		0.688	0.635	0.458	0.387	0.543	0.563		0.563	0.520	0.657	
	6×		0.250	0.271	0.177	0.105	0.160	0.229		0.375	0.172	0.271	
	8×		0.000	0.083	0.208	0.355	0.191	0.156		0.000	0.200	0.071	
	10×		0.000	0.000	0.083	0.065	0.000	0.000		0.000	*0.032*	0.000	
	2×		0.000	0.000	*0.010*[e]	0.000	0.000	0.000		0.000	*0.002*	0.000	
MinLm935-2	2×	58	0.792	0.802	0.625	0.508	0.670	0.760	0.452	0.531	0.660	0.929	0.454
	3×		0.125	0.156	0.333	0.371	0.319	0.240		0.219	0.292	0.071	
	6×		0.083	0.031	0.042	0.065	0.000	0.000		0.250	0.030	0.000	
	4×		0.000	0.010	0.000	0.056	0.011	0.000		0.000	*0.018*	0.000	
MinLm1139	5×	17	0.531	0.240	0.177	0.202	0.191	0.156	0.681	0.438	0.214	0.400	0.680
	7×		0.323	0.490	0.458	0.419	0.383	0.448		0.406	*0.436*	0.314	
	12×		0.115	0.229	0.302	0.306	0.372	0.344		0.094	0.294	0.257	
	3×		0.031	0.042	0.063	0.073	0.053	0.052		0.063	0.056	0.029	
MinLm2448	3×	90	0.969	0.979	0.990	0.968	0.968	1.000	0.041	0.906	0.980	1.000	0.073
	2×		0.031	0.021	0.010	0.032	0.032	0.000		0.094	0.020	0.000	
MinLm2451	3×	24	0.854	0.844	0.844	0.500	0.723	0.802	0.391	0.750	0.722	0.943	0.331
	5×		0.125	0.135	0.125	0.081	0.202	0.156		0.250	0.146	0.000	
	2×		0.021	0.021	0.031	0.419	0.074	0.042		0.000	0.132	0.057	

(Continues)

TABLE 3 (Continued)

Locus	Allele[a]	Repeat Motif (bp)	Allele frequency of year scale population						Gene diversity (h)[c]	Allele frequency of location scale population			Gene diversity (h)[c]
			pop10 96[b]	pop11 96	pop12 96	pop13 124	pop14 94	pop15 96		popAB 64	popMB 468	popSK 70	
MinLm2452	3×	21	0.927	0.969	0.938	0.863	0.894	0.958	0.142	0.844	0.916	1.000	0.151
	2×		0.042	0.000	0.052	0.097	0.096	0.042		0.063	0.064	0.000	
	15×		0.031	0.031	0.010	0.040	0.011	0.000		0.094	0.020	0.000	
H[d]			0.356	0.474	0.542	0.458	0.513	0.442		0.465	0.585	0.398	0.492

[a]Alleles scored as the number of repeat motifs.
[b]Number of isolates of the population.
[c]Mean gene diversity at each locus across nine subpopulations.
[d]Mean gene diversity in each subpopulation.
[e]Italised the different allele distribution compared to other subpopulations.

TABLE 4 Pairwise comparison of population Nei's genetic distance (F_{ST}) and Nei's genetic identity (*NeI*) Values in *Leptosphaeria maculans* populations in western Canada

Population	F_{ST} (*NeI*) Value for population: Year scale population						Location scale population			
	Pop10	Pop11	Pop12	Pop13	Pop14	Pop15		PopAB	PopMB	PopSK
Pop10	0.000	–	–	–	–	–	PopAB	0.000	–	–
Pop11	0.013 (0.987)[a]	0.000	–	–	–	–	PopMB	0.038 (0.962)	0.000	–
Pop12	0.029 (0.971)	0.023(0.977)	0.000	–	–	–	PopSK	0.085 (0.915)	0.058 (0.975)	0.000
Pop13	0.109 (0.891)	0.106 (0.894)	0.053 (0.947)	0.000	–	–				
Pop14	0.034 (0.967)	0.025 (0.975)	0.011 (0.989)	0.053 (0.947)	0.000	–				
Pop15	0.025 (0.975)	0.012 (0.988)	0.010 (0.990)	0.069 (0.931)	0.012 (0.988)	0.000				

[a]Indicates the genetic distance (F_{ST}) and identity (*NeI*) in *L. maculans* populations through pairwise comparison of each subpopulation.

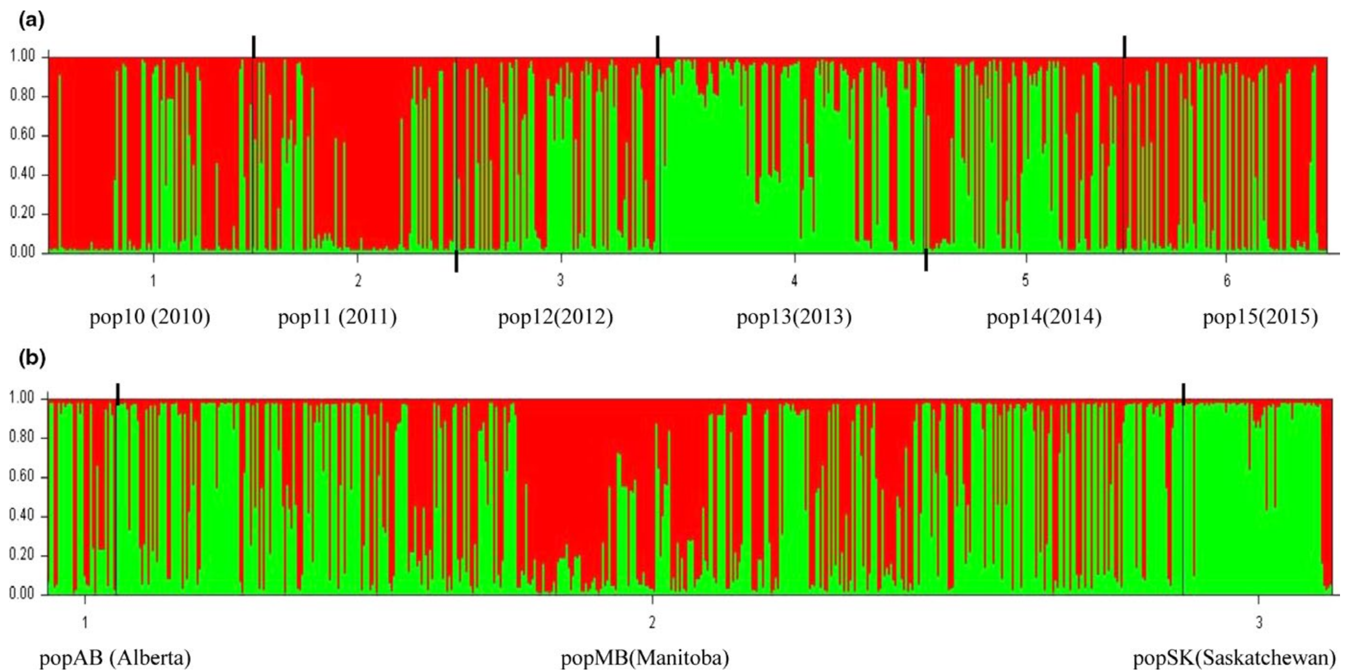

FIGURE 1 Population structure analysis showing the proportion of *Leptosphaeria maculans* isolates grouped by collection year (a) and location (b). Red and green colours indicate the population of cluster 1 and 2, respectively

TABLE 5 Isolate composition of *Leptosphaeria maculans* genetic clusters defined by high probability of membership value (*q*) using STRUCTURE

Population	Number of isolates	Inferred cluster 1	Inferred cluster 2	*K* value	Δ*K*
pop10	96	0.744	0.256	2	51.222
pop11	96	0.748	0.252	2	31.608
pop12	96	0.539	0.461	2	94.873
pop13	124	0.250	0.750	2	139.751
pop14	94	0.509	0.491	2	154.659
pop15	96	0.664	0.336	2	45.477
Year scale	602	–	–	2	39.795
popAB	64	0.453	0.547	2	56.245
popMB	468	0.482	0.518	2	74.256
popSK	70	0.131	0.869	2	61.203
Location sacle	602	–	–		98.974

genetic differentiations were mostly attributed to variations that occurred within each subpopulation.

3.5 | Genetic admixture analysis

Bayesian clustering of genetic admixture analyses using STRUCTURE Δ*K* values suggested that $K = 2$ is the optimal value of *K* (Figure 1, Figure S1) in both year and location subpopulations. The two clusters were well identified in all nine subpopulations (Table 5). With regard to the 6 year subpopulations, the membership values (*q*, proportion of the population) of cluster 1 were distributed from 0.250 to 0.748, with an average of 0.542 in the total population. However, the *q* values ranging from 0.518 to 0.869 were grouped in cluster 2 when the estimation was based on three location subpopulations. In contrast to the other 5 year subpopulations, most of the isolates ($q = 0.750$) were grouped in cluster 2 in the subpopulation of pop13 (Table 5).

4 | DISCUSSION

This study investigated the mating-type allele frequency and population diversity in *L. maculans* isolates collected from Alberta, Manitoba and Saskatchewan in 6 years. To the best of our knowledge, this is

the first study to investigate the genetic diversity, population structure and differentiation using minisatellite loci for genotyping of blackleg isolates collected in western Canada based on year and location subpopulations.

The hypothesis of random mating is based on the distribution of mating-type alleles in a 1:1 ratio within the field population of *L. maculans*. Our data show that both from within and between the different sampling years and/or locations, subpopulation isolates presented a 1:1 ratio distribution of the two mating-type alleles, suggesting that both mating-type alleles (*MAT1-1* and *MAT1-2*) in *L. maculans* have equal fitness and have no restriction of sexual recombination. However, it should be noted that asexual reproduction in the Canadian prairies has been shown to be the most common lifestyle among these isolates due to the environmental conditions, and cropping system (Dilmaghani et al., 2009; Ghanbarnia, Fernando, & Crow, 2011; Guo, Fernando, & Entz, 2005). Similarly, studies conducted in Australia and France (Barrins, Ades, Salisbury, & Howlett, 2004; Gout et al., 2006) also showed a distribution of mating-type allele ratio of 1:1; however, sexual recombination among these *L. maculans* isolates within these regions was shown to be more common.

The ten minisatellite loci evaluated in this study had a moderate number of alleles at each locus, ranging from two to six alleles, which enabled a population genetic analysis between the year and the location subpopulations. In contrast to the >40 alleles obtained in the six examined minisatellites or eight minisatellites and microsatellites in the France and Australia field populations of *L. maculans* (Gout et al., 2006; Hayden et al., 2007), respectively, the ten minisatellite loci examined in this study were all present with fewer alleles in each of the *L. maculans* isolates in western Canada. In addition, alleles detected in MinLm555, MinLm935-2, MinLm1139, MinLm2451 and MinLm2452 from western Canada field population of *L. maculans* were less than those detected in Poland (Jedryczka et al., 2010). However, two of the minisatellite loci, namely MinLm585 and MinLm2448, which did not generate polymorphic products in the *L. maculans* population from Poland (Jedryczka et al., 2010), were identified with six and two alleles in this study from the western Canada populations. Therefore, DNA polymorphisms identified by investigation of minisatellite loci in *L. maculans* isolates from the different geographical regions seem quite varied. Previous studies indicated that pycnidiospores were the main inoculum source for *L. maculans* infections, and fewer opportunities were present for sexual reproduction in isolates from western Canada due to the short cropping season and environmental conditions (Dilmaghani et al., 2009; Ghanbarnia et al., 2011; Guo et al., 2005). For example, pycnidia can survive the cold winters and release pycnidiospores at the next growing season, whereas ascospores cannot survive or are in a low concentration due to the repeated freezing and thawing during the long period of winter in western Canada. Ascospores are considered to play a major role as inoculum in blackleg epidemics in Australia (Barrins et al., 2004), France (Gout et al., 2006) and Poland (Jedryczka et al., 2010). The specificity of minisatellite polymorphisms identified in this study suggests that blackleg disease caused by *L. maculans* is probably subjected to selection pressure by the host and geographical differences in the regions, including environmental variations, or sexual/asexual reproduction difference. The 10 minisatellite loci selected were polymorphic from two to six alleles at each locus across the nine subpopulations. These populations had moderate gene diversity (H) of 0.492 (0.356–0.585 across subpopulations), which was higher than that of an *L. maculans* population from Mexico using minisatellites, or for a field population from Victoria, Australia (H = 0.12) using AFLP (Barrins et al., 2002; Dilmaghani et al., 2012). However, the gene diversity in the western Canada canola field *L. maculans* population was lower than that of the field population in France (H = 0.68–0.75) (Gout et al., 2006). The highest presence of alleles such as 4× of MinLm4 or 2× MinLm5 present in the field population of *L. maculans* from France was mostly consistent with the alleles of minisatellite identified in this study. The allele frequencies and the gene diversity of the nine subpopulations, despite being collected in six different years or from three different provinces, may have been coevolving in part in the genetic variation of a large population. Ascospores, usually considered to be airborne, which can survive for up to 6 weeks (Hammond & Lewis, 1986) and be dispersed over several kilometres, could be among the potential sources of genetic homogenization of the population. The geographical topography of the core agricultural provinces (Albert, Manitoba, and Saskatchewan) in western Canada, being clustered together and mostly flat, allows the relatively high exchange of gene flow via airborne spores. Therefore, the gene diversity of *L. maculans* isolates investigated in this study was lower than in European countries. With the high exchange of gene flow occurring over western Canada with larger growing areas of canola, a genetic drift can enable the *L. maculans* isolates collected in different locations over 6 years to develop different allele frequencies and divergence between populations.

The evidence of genetic differences between subpopulations from the three different provinces or from the 6 years was strengthened when population structures were analysed according to year and location. Two clusters with the highest probabilities were inferred from *L. maculans* isolates from the 6 year subpopulations and three location subpopulations. With the exclusion of pop13, the other 5 year subpopulations were assigned mostly to cluster 1. However, the three location subpopulations were mostly located in cluster 2 (Table 5). pop13, having been assigned to the admixed cluster 2, revealed similar structure analysis to that of popMB. The inferred cluster number (K = 2) of western Canada isolates is different from the previously reported result from Australia (K = 3) (Hayden et al., 2007). The difference is most likely a result of geographical/environmental differences between the two countries, as eastern and western regions of Australia are separated by the Nullabor plain, comprising nearly 1,200 km of arid to semi-arid desert which forms a barrier to gene flow. Whereas, isolates from western Canada with large growing areas of canola can support the gene drift caused by long distance dispersal of airborne spores.

A moderate level of differentiation (F_{ST}) between the subpopulations by year and location was demonstrated by AMOVA analysis.

The allele frequency and gene diversity indices analysed in this study were consistent with the results of population differentiation. Hierarchical analysis allowed the partitioning of the total genetic variation within and between the year and the location subpopulations. One finding of this study was that a large majority of the genetic differentiation was distributed within the year subpopulations and within the location subpopulations. The location subpopulation contributed a lower component of variation than that of the year population in the AMOVA analysis. Within subpopulations, genetic differentiation was associated with geographical distance and low F_{ST} values that are usually related to the subpopulations sampled from the same sites of western Canada. The lack of genetic differentiation between location subpopulations may be due to the long distance between the neighbouring three provinces, and the continuous growing of canola crops. Low frequency of gene flow may reduce the genetic differentiation by homogenizing the alleles of *L. maculans* in the field population. In western Canada, the large area devoted to canola production and tight crop rotations are among the contributing factors to the persistence of blackleg disease inoculum. Therefore, the population differentiation analysis is consistent with the previous evaluation of genetic diversity of *L. maculans* population, including the allele frequency, gene diversity, genetic differentiation analysis across each locus, and the population structure analysis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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