Genetic variation, genetic distance and population structure of the blackleg pathogen *Leptosphaeria maculans* of canola/rapeseed

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Abstract

Leptosphaeria maculans causes blackleg disease in canola (*Brassica napus* L.). The isolates can be identified into different pathogenicity groups based on the interaction phenotype on differential cultivars. PG-3, PG-4 and PGT strains were recently found in Western Canada and North Dakota (ND), USA. Genetic diversity and population structure of *L. maculans* population collected from a single field in La Riviere, Manitoba, Canada and other regions in ND, USA, Brazil, Australia and United Kingdom was investigated using the sequence-related amplified polymorphism (SRAP) marker technique. High number of polymorphic loci was found based on 91 polymorphic fragments and high number of genotypes was detected in each of those populations, suggesting that *L. maculans* is extensively diverse in genetics and each population consists of isolates with high number of unique genotypes. Phylogenic analysis indicated that all populations were clustered together. Indirect estimation of gene flow showed that high rate of gene flow existed among all populations with the greatest rate between La Riviere and ND (*Nm* = 15.96) as they are geographically close to each other. AMOVA revealed that a major genetic variance source came from the genetic variation among isolates within populations regardless of the origin and pathogenicity.

Key words: Genetic variation, population structure, blackleg, *Brassica napus*, *Leptosphaeria maculans*

Introduction

Blackleg, is the most destructive pathogen of canola/rapeseed worldwide. The presence of pathogenicity group 3 (PG-3) and 4 (PG-4) isolates has been detected for the first time since 2002 in Western Canada and North Dakota (ND), USA (Chen and Fernando, 2006; Bradley et al., 2005; Chen and Fernando, 2005; Fernando and Chen, 2003). Population structure and diversity of the isolates collected from above regions and from Brazil, Australia and United Kingdom were investigated using Sequence-Related Amplified Polymorphism (SRAP) molecular technique.

Materials and Method

A total of 116 isolates consisting PG-2, PG-3, PGT, PG-4 and *L. biglobosa* PG-1 were collected from six geographic regions, i.e. La Riviere and Roland in Manitoba, Canada, North Dakota in USA, Brazil, Australia and UK. Genomic DNA of each isolate was extracted with CTAB and four SRAP primer pairs, DC1/ODD30, FC1/ODD30, EM1/ODD30 and SA12/ODD30, were used for polymorphic amplification. PCR products were separated in an ABI Prism® 3100 Genetic Analyzer and analyzed using the GeneScan software. The polymorphic bands ranged from 100-600bp were scored using the Genographer program.

The analysis of molecular variance (AMOVA) within and among populations from six regions and five PGs was performed by treating a SRAP profile as a haplotype, using Arlequin version 3.0 software. A molecular evolutionary and phylogeny program, V_MLDIST, was used to compute maximum likelihood distances between SRAP discrete data of all 116 isolates and a consensus tree was constructed using Neighbor-Joining method with 100 bootstrap replicates. Population genetic analysis software programs POPGEN32 and TFPGA were used for allele frequency statistical analysis. The data were processed as a haploid model with two alleles per locus. Heterozygosity (*H*) and percent polymorphic loci (*r*) were estimated for geographic and PG populations. Genotypic diversity was calculated using Shannon's information index. Differentiation among populations from geographical locations and PGs was also estimated using an exact test (Raymond and Rousset, 1995) and by indirect estimation of gene flow using $Nm = ½ (1-G_{st})/G_{st}$ (Nei, 1973). A phenogram was constructed for PG populations using the unweighted pair-group method with arithmetic average (UPGMA) from a Nei's genetic distance matrix (Nei, 1978) in the TFPGA software package. Bootstrap sampling (1000 replicates) was performed for statistical support of branches of the constructed phenogram.

Results

Genetic Variation. A total of 91 polymorphic loci were generated from the 116 isolates using four pairs of SRAP primers. AMOVA analysis revealed that only 4.12% of variation was caused by differences among different geographic populations, and 95.88% was due to molecular diversity within population. AMOVA performed on PG populations showed that 10.82% of total variance was due to differences among PG groups, and 89.18% of total genetic variance was caused by differences within PG groups.

Genetic diversity assessed on geographic populations indicated that the number of polymorphic loci (*r*) varied from 54.95% of UK and Australia populations to 86.81% and 91.21% of La Riviere, Roland populations in Manitoba, Canada and North Dakota, USA. Based on the 91 polymorphic loci, the percentage of unique genotypes in geographic populations varied

from 71.43-100%. The average unbiased Heterozygosity (*H*) obtained by estimates of allele frequency according to Nei's (1978) formula varied from 22.51 % (Australia) to 32.6% (Roland). In general, all populations showed high levels of heterozygosity. Heterozygosity and genotypic diversity were also compared among five PG populations. A high level of polymorphic loci was shown in the isolates of PG-2 (86.81%), PG-3 (83.52%), PG-4 (72.53%) and PGT (58.24%) but not PG-1 (29.67%). Shannon index (*s*) indicated that the populations of PG-2 (0.42), PG-3(0.40), PG-4 (0.36) and PGT (0.29) were more diverse than those of PG-1 (0.17). With *H* values, a uniform level of heterozygosity and genotypic diversity was observed in the populations of PG-2, PG-3, PG-4 and PGT ($H > 20.0\%$), but PG-1 ($H = 13.0\%$). In general, all isolates in the different PGs showed high levels of unique genotypes (100% in all PGs).

Population structure. SRAP data did not show a high level of discrimination between the geographic populations but PG populations. Genetic identity and distances were not significantly different among 15 pair combinations of six geographic populations. Genetic identity, as presented by Nei's genetic identity index (Nei, 1978), ranged from the maximum value 0.99 (between the La Riviere and ND populations) to the minimum value 0.90 (between the Australian and Roland populations). Nei's (1978) genetic distance coefficients were ranged from the maximum value 0.11 to the minimum value 0.01 and no big difference was observed. A dendrogram constructed with program V_MLDIST to assess relatedness among 116 isolates showed that 91% of isolates, no matter of geographic or PG origin, was clustered together and no distinct subgroups was capable of differentiating geographic populations. However, all PG-1 isolates were classified together as a distinct subgroup. Exact tests used for analyzing population differentiation didn't show significant differentiation between the populations as all *p* values > 0.05. Indirect estimation of gene flow between geographic populations using the number of migrants (*Nm*) as a measurement of population differentiation showed that the rates of gene flow between populations ranged from 2.77 to 15.96 migrants per generation, meaning that gene migration was quite different between population pairs. The lowest gene flow occurred between Australian and other populations (Nm =2.77-5.46). Populations between Australia and United Kingdom also had lower gene flow ($Nm = 3.82$). Higher probabilities of gene flow seem to have taken place among La Riviere, Roland, North Dakota, Brazil, and UK populations (Nm = 6.42 to 15.96). In general, when higher gene flow was observed, lower differentiation among population pairs from each other was observed. For instance, lowest differentiation and greatest gene flow were measured between the La Riviere and North Dakota populations ($Nm = 15.96$, $P = 0.30$).

Population structure analyzed for PG groups indicated that Nei's (978) genetic distances among five PG populations ranged from 1.17 to 35.70%. Based on the genetic identity and distances, the populations from the PG-2 and PG-3, PG-4 and PGT (Nei's distance < 5.0%) were more closely related to each other than to PG-1 population (Nei's distance > 28.1%). A UPGMA dendrogram generated for PG populations using Nei's' (1978) unbiased genetic distances indicated that PG-1 isolates were closely clustered into one subgroup, it was obviously different from other PG isolates which were fallen into another distinct subgroup with statistically well supported branches by 1000 bootstrappings. Significant differentiation was also detected between population pairs from different PG populations based on the exact tests. The rate of gene flow for populations from different PGs was highly different. PG-1 population had very low gene flow to other PG populations ($Nm <$ 1). But high gene flow was observed among PG-2, PG-3, PG-4 and PGT ($Nm > 5.2$).

Discussion

The frequency of recombination through the sexual stage is an important genetic diversity source in fungal populations. In this study, we found that a high level of genetic diversity existed in the *L. maculans* and *L. biglobosa* populations. In all six populations, nearly every isolate has unique genotype, suggesting that a high level of recombination had occurred in these populations, thus causing a high level of genetic diversity.

Populations of *L. maculans* from Australia and United Kingdom had lower genetic diversity compared to the populations from North America and Brazil consisting of *L. maculans* and *L. biglobosa*. However, this didn't result in different genetic clusters. One of the factors responsible for this may be that populations from Australia and United Kingdom were geographically and genetically distant, but the North American populations shared markers with both regions (Purwantara et al., 2000). Disparity of PG structure in the populations could be another reason for differences (Balesdent et al., 2005) because the populations collected from Australia and United Kingdom was less diverse in the structure of PGs (absence of PG-1 isolates).

Our results showed that among six populations, there existed high levels of gene flow that most isolates from different locations were clustered together. However, the rate of gene flow among the populations was different. It seems like populations from closer geographic locations had higher possibility of gene exchange. This result supported the conclusion by Pongam et al (1999) that *L. maculans* was introduced into North Dakota from Western Canada. According to Gout et al. (2006), the reason of a high level of gene flow between the populations with short distance could be proposed as either natural long distance dispersal of propagules within a country or a rapid countrywide diffusion of novel virulence alleles whenever novel resistance sources are used. Relative lower level of gene flow was found among populations from Australia and United Kingdom ($Nm = 3.82$), as they were separated in geographic locations, which supported Purwantara's results (2000). However, gene flow did occur between these two populations as $Nm > 1$. This could be proposed as human activities leading to genetic exchange between populations located on different continents.

The single field population from La Riviere which was severely infected by blackleg in 2003 is a good example to demonstrate that sexual recombination and human activities may have caused genetic alternation. It was found that this population had the most divergence in PG constitution (PG2 to PGT), high number of genotype ($r = 86.81$), high proportion of heterozygosity ($H = 0.27$), and gene flow to the Australia ($Nm = 5.45$) and United Kingdom ($Nm = 8.85$). The isolates in this

population was not clustered into different groups with ones from Australia and United Kingdom populations, indicating that the global gene flow existed in *L. maculans* populations.

Pathogenicity group is an important index of pathogen aggressive ability (Mengistu et al., 1991). Among the PG populations, PG-1 had the lowest genotype (r = 29.67%) and heterozygosity (H = 0.13) compared with other PG populations, and also had a much greater genetic distance (> 28.13%) from other PG populations, which made it fall into a different cluster from PG-2, PG-3, PG-4 and PGT. This result showed that the PG-1 population was different from other PG populations in genetics and also resulted in PG-1 being assigned as a new species *L. biglobosa* (Shoemaker and Brun, 2001). No significant genetic difference among PG-2, PG-3, PG-4 and PGT populations were observed. The rate of gene flow among these PG groups was high (Nm > 5.21), which decreased the genetic diversity among them. The gene flow between PG-1 and other PGs was very low (Nm < 1) that led to large genetic diversity (Nei's genetic distance > 0.28) between them. The new North American PG-3 population, which was recently observed in Western Canada and North Dakota, was compared with PG-3 populations from Australia and United Kingdom. The result indicated that the North American PG-3 population was genetically similar to Australia's and United Kingdom's PG-3 (P > 0.05), suggesting that PG-3 found in Western Canada and North Dakota had most likely evolved through either sexual recombination but possibly imported from Europe or from eastern Canada because the isolates between eastern Canada (Ontario) and Europe were similar (Balesdent et al., 2005). However, further research is needed before stronger conclusions can be made with respect to the origin of PG-3, PG-4 and PGT found in Western Canada and North Dakota.

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