Microsatellite and Minisatellite Analysis of *Leptosphaeria maculans* in Australia Reveals Regional Genetic Differentiation

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ABSTRACT

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The population genetic structure of the fungal pathogen *Leptosphaeria maculans* was determined in Australia using six microsatellite and two minisatellite markers. Ascospores were sampled from *Brassica napus* stubble in disease nurseries and commercial fields in different sites over 2 years. The 13 subpopulations of *L. maculans* exhibited high gene ($H = 0.393$ to 0.563) and genotypic diversity, with 357 haplotypes identified among 513 isolates. Although the majority of genetic variation was distributed within subpopulations (85%), 10% occurred between the regions of eastern and Western Australia, and 5% within regions. F_{ST} analysis of subpopulation pairs also showed the east-west genetic differentiation,

whereas factorial correspondence analysis separated Western Australian subpopulations from eastern ones. Bayesian model-based population structure analyses of multilocus haplotypes inferred three distinct populations, one in Western Australia and an admixture of two in eastern Australia. These two regions are separated by 1,200 km of arid desert that may act as a natural barrier to gene flow, resulting in differentiation by random genetic drift. The genetic differentiation of *L. maculans* isolates between eastern and Western Australia means that these regions can be treated as different management units, and reinforces the need for widespread disease nurseries in each region to screen breeding lines against a range of genetic and pathogenic populations of *L. maculans*.

Additional keywords: blackleg, *Phoma lingam*, Phoma stem canker.

Leptosphaeria maculans (Desm.) Ces. & De Not. (anamorph = *Phoma lingam* (Tode) Desm.) is a heterothallic ascomycete that causes blackleg or Phoma stem canker of *Brassica napus* L. var. *oleifera* (canola, oilseed rape, and colza) worldwide (18,43). The lifecycle consists of a single sexual generation per season that develops on infected *B. napus* stubble during summer, followed by several asexual generations during the growing season of the crop, which is from June or July to December in Australia. Ascospores infect seedlings via the stomatal aperture or wounds and produce pycnidia in lesions that develop within ≈ 10 days after penetration (13). Conidia then act as secondary inoculum being splash-dispersed onto other leaves and neighboring plants. Hyphae grow from the leaf lesions down the vascular tissue of the petiole into the stem, then the crown, eventually producing a black stem canker and girdling the stem (14). After plant death, the fungus colonizes the stubble, and sexual recombination results in the formation of pseudothecia. Vegetative fruiting bodies (pycnidia) also form directly on stubble.

L. maculans occurs almost everywhere *B. napus* is grown, causing significant yield losses in Australia, Europe, and Canada (43). The disease has spread primarily as a result of worldwide seed transmission (9), with epidemics reported in France in 1950 (8), Germany in the 1960s (22), the United Kingdom in 1977 (10), and Canada in 1975 (29). *B. napus* production commenced in Australia in 1969 using Canadian spring cultivars that had no blackleg resistance. By 1971, blackleg epidemics had such a dramatic impact on Australian crops that very little *B. napus* was grown over the next 10 to 15 years (6,28). Australian-adapted

cultivars with blackleg resistance conferred by several genes (polygenic resistance) became available in the early 1980s when the industry began to recover, before expanding rapidly in the 1990s (39).

Genetic diversity of *L. maculans* has been examined at different geographical scales; for example, between leaves and plants within a field (5,16), between fields within a country (4,12,23), and across continents (37), using a variety of genetic markers. Differing scenarios have been described for the population structure at the macrogeographic scale. In Canada, isolates from two fields were shown to belong to two genetically distinct populations (23), while isolates from different fields in France and Australia each belonged to one population (4,12). One reason for these different scenarios is that different populations of the same species can have extremely different structures, due to variation in allele frequencies caused by genetic drift, restricted gene flow, founder effects, or selection. Also, overall genetic structure of populations may be very different due to the extent of recombination, which affects the multilocus structure of populations (32). The latter case is unlikely for *L. maculans*, given that recombination is such an important evolutionary force in the genetic structure of populations (4,12,16,23). The previous study of the population structure of *L. maculans* in Australia mainly used fingerprinting markers in the form of amplified fragment length polymorphisms (AFLPs) and examined only a small number of populations (4). The study described in this article examines a greater number of isolates from more dispersed geographical sites using single-copy markers as measures of genetic diversity (26).

Knowledge of genetic structure of pathogen populations can help optimize screening procedures in plant breeding programs by ensuring that *B. napus* lines are exposed to all known pathogen variation, while selecting the most effective and enduring resistance (31). In Australia, a range of management practices is used

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to achieve this. Sometimes disease nurseries are established upon stubble remaining from a commercial crop. In other cases, the nursery might be in a similar location each year and exposed to stubble from a single cultivar, or left behind from the previous nursery screening with a mix of cultivars harboring the strains that had infected plants during the previous year. Effective screening for blackleg resistance is of particular importance for *L. maculans* in Australia because, recently, the effectiveness of resistance in *B. napus* cultivars with major gene resistance derived from *B. rapa* subsp. *sylvestris* (hereafter referred to as “sylvestris-derived” cultivars) declined dramatically only 3 years after commercial release of the cultivars (41). Furthermore, Australian isolates of *L. maculans* are extremely diverse in terms of their avirulence (*Avr*) allele combinations, comprising all known existing virulence and avirulence alleles. Of the 26 races identified in a global collection of *L. maculans*, 18 were defined by Australian isolates (3). Information on the distribution of genetic diversity in *L. maculans* and level of gene flow has implications for disease management issues such as new virulence allele combinations and how these may spread among populations.

Fig. 1. Sites of the 13 subpopulations of *Leptosphaeria maculans* collected from disease nurseries and commercial fields of *Brassica napus* throughout New South Wales (NSW), Victoria (VIC), South Australia (SA), and Western Australia (WA) between mid-2002 and mid-2003. Two commercial fields (2.5 km apart) were sampled at Thuddungra, NSW. The same location was sampled in both years at Struan, SA and Mt. Barker, WA.

The objectives of this research were to determine the population structure of *L. maculans* in Australia and follow changes in the population structure through time, and to estimate the genetic differentiation among samples of *L. maculans* from disease nurseries and commercial *B. napus* fields.

MATERIALS AND METHODS

Sampling. A total of 513 isolates of *L. maculans* was collected from infected stubble of several *B. napus* cultivars. This stubble was collected in mid-2002 and mid-2003 from blackleg disease nurseries, a cultivar trial site, and commercial fields at 11 sites in Victoria (VIC), New South Wales (NSW), South Australia (SA), and Western Australia (WA) (Fig. 1; Table 1). Blackleg disease nurseries are used to evaluate seedling and adult plant resistance in *Brassica* cultivars under high *L. maculans* inoculum pressure. Blackleg disease nurseries at the same location were sampled in both years at Mt. Barker, (WA) and Struan (SA). The Mt. Barker nursery was rotated around different fields each year, with the new nursery crop sown into stubble of a polygenic cultivar, while the Struan nursery was sown back into the same field, which contained stubble of a mixture of cultivars with polygenic or sylvestris-derived major gene resistance. The Lake Bolac (VIC) nursery was sown into the stubble of a commercial field in different sites each year, while the Wonwondah nursery also was sown into the stubble of a commercial field. The cultivar trial at Edillilie (SA) was a site where the yield of a range of plant lines is compared with that from commercial cultivars. The plants are not grown on *B. napus* stubble but in a normal cropping rotation (one crop in every 3 or 4 years) for the district. For this reason, the sample from the cultivar trial site is considered as a commercial field and referred to as such hereafter in this article.

Stubble collected from commercial fields in NSW and disease nurseries in VIC and WA most commonly came from cv. TI1 Pinnacle, which has an intermediate level of polygenic resistance to *L. maculans*, as well as cvs. Grace, ATR-Beacon, and Hyden, which have high levels of polygenic resistance. Stubble collected from the Struan disease nursery and Edillilie trial site came from a mixture of cultivars, with the majority having polygenic resistance, whereas some had major gene sylvestris-derived resistance. A maximum of 14 pieces of stubble was sampled per location, and several single ascospores were isolated per stubble piece. A total of 205 isolates was obtained in 2002 and 308 in 2003. The 13 samples (Table 1) collected from 11 geographic locations over the 2-year period henceforth are referred to as subpopulations. The isolation of single ascospores, culturing, and DNA extraction were as described previously (16).

Microsatellite and minisatellite markers. Eight markers were used to examine genetic diversity. These included six micro-

TABLE 1. Sampling information for the 13 subpopulations of *Leptosphaeria maculans* from New South Wales (NSW), Victoria (VIC), South Australia (SA), and Western Australia (WA) in Australia analyzed in this study

Subpopulation	Sites	Type of <i>Brassica napus</i> field	<i>B. napus</i> cultivar ^a	Sample size	No. of stubble pieces	Collection date
STR-02	Struan, SA	Disease nursery	Cultivar mixture	40	6	2002
TH1-02	Thuddungra, NSW	Commercial field 1	TI1 Pinnacle	30	8	2002
TH2-02	Thuddungra, NSW	Commercial field 2	Grace	38	4	2002
MTB-02	Mt Barker, WA	Disease nursery	TI1 Pinnacle	79	9	2002
LA1-02	Lake Bolac, VIC	Disease nursery 1	TI1 Pinnacle	18	3	2002
STR-03	Struan, SA	Disease nursery	Cultivar mixture	42	13	2003
EDI-03	Edillilie, SA	Cultivar trial	Cultivar mixture	23	4	2003
LA2-03	Lake Bolac, VIC	Disease nursery 2	TI1 Pinnacle	36	10	2003
WON-03	Wonwondah, VIC	Disease nursery	ATR-Beacon	37	14	2003
MTB-03	Mt Barker, WA	Disease nursery	TI1 Pinnacle	59	12	2003
WOH-03	Wongan Hills, WA	Disease nursery	Hyden	45	6	2003
LOC-03	Lockhart, NSW	Commercial field	ATR-Beacon	46	11	2003
GRE-03	Grenfell, NSW	Commercial field	TI1 Pinnacle	20	2	2003
Total	...	...	...	513	102	...

^a Cultivar mixture = stubble was from several cultivars with polygenic resistance or major gene resistance derived from *B. rapa* subsp. *sylvestris*.

satellite loci (*Lema* 4, *Lema* 6, *Lema* 12, *Lema* 15, *Lema* 34, and *Lema* 39) (16,17), the minisatellite locus *MinLm1* (2), and an additional minisatellite locus, *Lema* 37 (GenBank accession no. AY916774), with a core motif of (GGATTGGGT)₂N₂₁(GGATTGGGT)₂. Primers *Lema* 37F (5'-TCTTGGCTTGGCTTTG-TCTT-3') and *Lema* 37R (5'-ACATTGGGCTCGGAACATTC-3') were designed to amplify this locus, which was based upon DNA sequence data from a cosmid clone (19). Eight alleles were observed for this locus, and their sizes and repeat motifs are listed in Table 2. Microsatellite and minisatellite fragments were analyzed as described previously (16).

Data analysis. Multilocus haplotypes (MLHT) were constructed for each isolate by compiling the alleles at all loci. Isolates with the same MLHT were considered clones. Clone-corrected allele frequencies were estimated for each subpopulation at the eight loci using FSTAT (Version 2.9.3 for Windows; Department of Ecology and Evolution, Faculty of Biology and Medicine, University of Lausanne, Switzerland), and used for the analysis of gene diversity and population differentiation. Nei's gene diversity (*H*) (33) was estimated at each locus and within each subpopulation (averaged across loci).

Isolates with missing data due to lack of amplification at a locus were excluded from the genotypic diversity calculations; hence, only 495 of the 513 isolates were analyzed. Genotypic diversity (*D*) was calculated using $D = (n/n - 1)(1 - \sum p_i^2)$ as implemented in MULTILOCUS (version Win 1.3b; Department of Biology, Imperial College, Silwood Park, United Kingdom), where p_i is the frequency of the *i*th genotype and *n* is the number of individuals sampled. *D* represents the probability that two individuals, sampled at random with replacement, have the same genotype and is similar to the diversity measure of Pielou (36).

The hypothesis of random mating within subpopulations was tested using two approaches with clone-corrected data sets. Firstly, a Fisher exact test was used to test for departure from gametic equilibrium using GENEPOP (version 3.1b; Centre d'Ecologie Fonctionnelle et Evolutive, Centre National de la Recherche Scientifique, Montpellier, France) as described previously (16). Second, associations among loci were tested using the index of multilocus gametic disequilibrium $\bar{r}_d$ (1) implemented using MULTILOCUS. The index $\bar{r}_d$ has an expected value of zero if there is no association of alleles at unlinked loci, as expected in a randomly mating population. The significance of $\bar{r}_d$ was tested by comparing the observed variance with the distribution of the variance expected under the null hypothesis of random mating, as determined from 1,000 randomizations of the haplotype data. Isolates with missing data were not included in the index of multilocus gametic disequilibrium calculations.

Genetic differentiation was examined using four different techniques between clone-corrected subpopulations from different sites in two regions, eastern (NSW, VIC, and SA) and Western Australia (WA), over 2 years, and between disease nurseries and commercial fields. First, a hierarchical analysis of molecular variance (AMOVA) was estimated using ARLEQUIN (version 3.01; Zoological Institute, Department of Biology, University of Bern), whereby the partitioning of genetic variability was examined

among regions (east versus west), among subpopulations within a region, and within subpopulations.

Second, the parameter F_{ST} was estimated to examine pairwise differences between subpopulations separated spatially and temporally, and between the types of sites sampled. Temporal differentiation was tested between groups of subpopulations from 2002 and 2003, and between subpopulations from Struan (SA) and Mt. Barker (WA), where samples were collected from the same location both years. Genetic differentiation was determined between disease nurseries (STR-02, MYB-02, LA1-02, STR-03, LA2-03, WON-03, MTB-03, and WOH-03) and commercial fields (TH1-02, TH2-02, LOC-03, GRE-03, and EDI-03). The null hypothesis of no differentiation between subpopulations was tested using FSTAT to estimate $\hat{\theta}$ (42), an unbiased estimator of the parameter F_{ST} . Significance levels were determined after strict (not sequential) Bonferroni corrections based on the indicative adjusted *P* value following 78,000 permutations. Genetic differentiation between subpopulations at a locus was considered significant when *P* was less than the 5% nominal level.

Third, factorial correspondence analysis (FCA) was performed to depict the influence of specific subpopulations or alleles on the overall pattern of genetic variance using GENETIX (version 4.05.2; Laboratoire Génome, Populations, Interactions, Université Montpellier II, France), based upon the allele present at each locus for each isolate. Correspondence analysis maximizes the correspondence between the alleles scores and subpopulation scores. First-axis allele scores and subpopulation scores are assigned such that the weighted correlation between the two is maximized where the "weight" is the frequency of the allele. The other axes similarly represent the correlation coefficient between the allele and subpopulation scores, though at lower levels of variance.

Fourth, population structure also was examined independently of the geographic location of sampled individuals to estimate the number of populations (*K*) and to assign individuals probabilistically to "genetic" populations using multilocus haplotype data. This was done using a Bayesian clustering approach implemented in STRUCTURE (version 2.1; Department of Human Genetics, University of Chicago, Chicago). Because the program assumes linkage equilibrium among loci within populations, any loci at disequilibrium were removed from the data set. The admixture model was evaluated, which assumes gene flow among populations. Analyses consisted of five independent simulations for each *K* with 10⁵ burn-in replicates and a run length of 10⁵ replicates, and assumed correlated allele frequencies. Log likelihood values and posterior probabilities ($P[K|X]$) were estimated assuming one to eight population clusters (*K* = 1, 2...8).

Additionally, isolation by distance between the subpopulations was tested using the method of Rousset (38), under which a correlation is expected between the logarithm (in a two-dimensional environment) of the geographical distance and Rousset's genetic distance $F_{ST}/(1 - F_{ST})$. This correlation was tested using matrix correlation methods based on a Mantel test using the program IBD (version 1.52; Department of Biology, San

TABLE 2. Characterization of the minisatellite alleles of the locus *Lema* 37 examined in *Leptosphaeria maculans* populations from New South Wales (NSW), Victoria (VIC), South Australia (SA), and Western Australia (WA)

Allele	Isolates sequenced	Size (bp)	Repeat motif
1	LM270	157	(GGATTGGGT) ₂
2	LM224, LM225	166	(GGATTGGGT) ₃
3	LM322	187	(GGATTGGGT) ₁ N ₂₁ (GGATTGGGT) ₂
4	LM214, LM251	196	(GGATTGGGT) ₂ N ₂₁ (GGATTGGGT) ₂
5	LM187, LM189	205	(GGATTGGGT) ₃ N ₂₁ (GGATTGGGT) ₂
6	LM188, LM190	235	(GGATTGGGT) ₂ N ₂₁ (GGATTGGGT) ₂ N ₂₁ (GGATTGGGT) ₂
7	LM186, LM192	244	(GGATTGGGT) ₃ N ₂₁ (GGATTGGGT) ₂ N ₂₁ (GGATTGGGT) ₂
8	LM385, LM388	283	(GGATTGGGT) ₃ N ₂₁ (GGATTGGGT) ₂ N ₂₁ (GGATTGGGT) ₂ N ₂₁ (GGATTGGGT) ₂

Diego State University, San Diego, CA). The geographic distance between two sampling points was computed as a linear distance. Isolation by distance was tested over all subpopulations in the study, and among subpopulations within a region (east and west).

RESULTS

Genetic diversity within populations. A total of 43 alleles was found at the six microsatellite and two minisatellite loci examined in 513 isolates. The 13 subpopulations of *L. maculans* had similar alleles at most loci, though the most frequent allele differed amongst the subpopulations at six of the loci (Table 3).

For example, loci *Lema* 4 and *Lema* 12 had four and three different alleles, respectively, as the most frequent in a subpopulation (alleles 1, 2, 3, and 4 for *Lema* 4 and 3, 6, and 7 for *Lema* 12), with these frequencies ranging from 0.308 to 0.724 at the two loci (Table 3). Changes were observed in the allele frequencies within a subpopulation at Struan in SA and Mt. Barker in WA during the 2 years. Gene diversity was moderate to high for the 13 subpopulations, ranging from $H = 0.393$ to 0.563 . Gene diversity for the total population was $H = 0.701$ over seven loci. Locus *Lema* 15 was excluded from the analysis because it was polymorphic in only 1 of the 13 subpopulations (LOC-03) (Table 3).

TABLE 3. Allele frequencies, gene and genotypic diversity of the 13 Australian subpopulations of *Leptosphaeria maculans* at six microsatellite and two minisatellite loci

Locus, allele ^a	Subpopulations ^b													H^c
	STR-02 (29)	TH1-02 (26)	TH2-02 (37)	MTB-02 (66)	LA1-02 (14)	STR-03 (39)	EDI-03 (15)	LA2-03 (29)	WON-03 (33)	MTB-03 (46)	WOH-03 (35)	LOC-03 (42)	GRE-03 (14)	
<i>Lema</i> 12														
1	0.000	0.308	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.745
2	0.000	0.000	0.000	0.000	0.000	0.000	<u>0.067</u>	0.000	0.000	0.000	0.000	0.000	0.000	...
3	0.724	0.462	0.459	0.182	0.143	0.538	0.533	0.621	0.424	0.043	0.057	0.310	0.571	...
4	0.000	0.038	0.081	0.015	0.000	0.000	0.000	0.172	0.091	0.043	0.114	0.119	0.429	...
5	0.034	0.000	0.135	0.000	0.000	0.179	0.067	0.034	0.030	0.000	0.086	0.143	0.000	...
6	0.069	0.077	0.054	0.485	0.000	0.154	0.200	0.103	0.303	0.522	0.457	0.286	0.000	...
7	0.172	0.115	0.270	0.288	0.857	0.103	0.133	0.069	0.152	0.391	0.229	0.143	0.000	...
8	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.057	0.000	0.000	...
<i>Lema</i> 15														
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.952	1.000	0.007
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	<u>0.048</u>	0.000	...
<i>Lema</i> 39														
1	0.517	0.923	0.757	0.758	0.643	0.897	0.733	0.828	0.848	0.761	0.829	0.786	0.714	0.357
2	0.483	0.077	0.243	0.242	0.357	0.103	0.267	0.172	0.152	0.239	0.171	0.214	0.286	...
<i>MinLm1</i>														
1	0.276	0.654	0.730	0.106	0.143	0.590	0.067	0.621	0.424	0.043	0.114	0.690	0.643	0.723
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	<u>0.071</u>	...
3	0.000	0.115	0.054	0.152	0.000	0.051	0.000	0.034	0.091	0.109	0.171	0.024	0.000	...
4	0.207	0.115	0.189	0.636	0.000	0.282	0.667	0.172	0.242	0.674	0.600	0.119	0.071	...
5	0.276	0.000	0.000	0.076	0.143	0.077	0.133	0.034	0.152	0.109	0.086	0.119	0.000	...
6	0.241	0.115	0.027	0.030	0.714	0.000	0.133	0.138	0.091	0.022	0.029	0.048	0.214	...
<i>Lema</i> 34														
1	0.000	0.038	0.054	0.061	0.000	0.026	0.000	0.000	0.061	0.087	0.000	0.071	0.000	0.552
2	0.000	0.385	0.270	0.667	0.143	0.462	0.133	0.414	0.485	0.609	0.829	0.310	0.357	...
3	0.931	0.577	0.649	0.227	0.786	0.436	0.867	0.517	0.394	0.304	0.171	0.571	0.643	...
4	0.000	0.000	0.000	0.030	0.071	0.077	0.000	0.034	0.061	0.000	0.000	0.048	0.000	...
5	0.000	0.000	0.000	<u>0.015</u>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	...
6	0.069	0.000	0.027	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	...
<i>Lema</i> 37														
1	0.000	0.000	<u>0.027</u>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.713
2	0.069	0.000	0.027	0.061	0.000	0.103	0.000	0.000	0.000	0.043	0.000	0.071	0.000	...
3	0.000	0.000	0.000	<u>0.015</u>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	...
4	0.034	0.115	0.216	0.242	0.071	0.179	0.267	0.103	0.212	0.217	0.429	0.143	0.429	...
5	0.483	0.269	0.297	0.227	0.929	0.282	0.267	0.483	0.242	0.217	0.171	0.333	0.000	...
6	0.069	0.038	0.027	0.015	0.000	0.026	0.000	0.034	0.091	0.000	0.000	0.095	0.071	...
7	0.345	0.577	0.405	0.303	0.000	0.385	0.333	0.379	0.455	0.478	0.343	0.357	0.500	...
8	0.000	0.000	0.000	0.136	0.000	0.026	0.133	0.000	0.000	0.043	0.057	0.000	0.000	...
<i>Lema</i> 6														
1	0.000	0.000	0.000	0.000	0.214	0.000	0.000	0.000	0.030	0.000	0.000	0.024	0.000	0.536
2	0.793	0.654	0.459	0.606	0.214	0.667	0.933	0.690	0.394	0.761	0.886	0.667	0.571	...
3	0.000	0.154	0.243	0.152	0.571	0.128	0.067	0.172	0.212	0.130	0.029	0.238	0.071	...
4	0.207	0.192	0.297	0.242	0.000	0.205	0.000	0.138	0.333	0.109	0.086	0.071	0.357	...
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	...
<i>Lema</i> 4														
1	0.000	0.000	0.054	0.045	0.429	0.154	0.000	0.103	0.212	0.043	0.000	0.095	0.143	0.736
2	0.034	0.231	0.432	0.258	0.286	0.308	0.400	0.379	0.364	0.304	0.171	0.405	0.071	...
3	0.241	0.385	0.324	0.500	0.143	0.256	0.467	0.345	0.303	0.348	0.314	0.310	0.429	...
4	0.690	0.269	0.189	0.182	0.143	0.231	0.133	0.172	0.091	0.261	0.486	0.167	0.286	...
5	0.034	0.115	0.000	0.015	0.000	0.051	0.000	0.000	0.030	0.043	0.029	0.024	0.071	...
H_T	0.420	0.477	0.513	0.513	0.393	0.509	0.435	0.487	0.563	0.481	0.435	0.535	0.497	...
D	0.982*	0.991 ^{NS}	0.999 ^{NS}	0.997 ^{NS}	0.979 ^{NS}	0.999 ^{NS}	0.977*	0.991 ^{NS}	0.997*	0.992*	0.986*	0.999 ^{NS}	0.947***	...

^a H_T = mean gene diversity in each subpopulation (34) and D = genotypic diversity (1). Null hypothesis of $D = 1$ was tested (one-sided test) using the number of D values from 1,000 randomized data sets which were smaller than the D value estimated from the data set; ^{NS} = $P > 0.05$, * = $P < 0.05$, and *** = $P < 0.001$.

^b Numbers in parentheses indicate clone-corrected sample size. Frequencies of alleles that occur in only one population (private alleles) are underlined.

^c Mean gene diversity at each locus across the 13 subpopulations (34).

In all, 357 unique multilocus haplotypes were found among the 495 isolates that had no missing data, with 105 haplotypes occurring at least twice. Within each subpopulation, the number of unique haplotypes ranged from 14 to 66. A few putative clones were distributed among and within the 13 subpopulations of *L. maculans*. These putative clones had different genotypes when primers for fingerprinting (20) and mating type idiomorph (7) were used (data not shown). Genotypic diversity (D) ranged from 0.947 to 0.999, and was significantly <1 ($P < 0.001$) in only one subpopulation (GRE-03) and the total population (Table 3).

Gametic disequilibrium. Gametic disequilibrium was tested at eight loci for clone-corrected data sets. The Fisher exact test detected low levels of disequilibrium for 7 (STR-02, TH2-02, MTB-02, LA2-03, WON-03, MTB-03, and GRE-03) of the 13 subpopulations in the locus-locus comparisons (data not shown). The index of multilocus gametic disequilibrium was low in all subpopulations, ranging from $\bar{r}_d = -0.011$ to 0.055, with the null hypothesis of random mating rejected for six subpopulations (STR-02, TH2-02, WON-03, LA2-03, LOC-03, and GRE-03) at $P < 0.05$ and one subpopulation at $P < 0.001$ (MTB-02) (data not shown). The loci pairs were examined in each of these clone-corrected subpopulations because the Fisher exact test showed only one loci pair at disequilibrium. The locus *Lema* 39 was present in three of the four different loci pairs, with loci pair, *Lema* 39 $\times$ *MinLm1*, occurring in four of the seven subpopulations at disequilibrium. Removing locus *Lema* 39 from the clone-corrected data sets of subpopulations STR-02, TH2-02, MTB-02, LA2-03, WON-03, MTB-03, and GRE-03 resulted in no loci pairs at gametic disequilibrium for all subpopulations using the Fisher exact test and, also, with the exception of LA2-03, where the loci pair at disequilibrium was *Lema* 12 $\times$ *Lema* 37.

Genetic differentiation among populations. Hierarchical AMOVA indicated that 10% of the total variance was distributed among the regions of eastern Australia (NSW, VIC, and SA) and Western Australia (WA), with 5% among subpopulations within a region and 85% within subpopulations. This east-west genetic differentiation was consistent with the F_{ST} analyses, where moderate levels of significant genetic differentiation were found between the regions, with $F_{ST} = 0.130$ and 0.106 ($P < 0.001$) in 2002 and 2003, respectively.

The F_{ST} analyses revealed a wide range of genetic differentiation levels between subpopulation pairs, depending on the distance between their sites and the year of sampling (Table 4). Between subpopulations within the same state (NSW, VIC, SA, and WA) and eastern Australia, F_{ST} estimates generally were low and nonsignificant, ranging from $F_{ST} = 0.001$ to 0.095. For example, there was no genetic differentiation ($F_{ST} = 0.017$, $P > 0.05$) between the closest subpopulations, TH1-02 and TH2-02, sampled from farms only 2.5 km apart in NSW (Table 4). There

was significant differentiation between subpopulations from more distant states; for example, WA and VIC, SA and NSW, and SA and WA, with F_{ST} estimates significant at the 5% level ($F_{ST} = 0.051$ to 0.097) and 1% level ($F_{ST} = 0.046$ to 0.155). Excluding subpopulations LA1-02 or STR-02, the greatest genetic differentiation ($F_{ST} = 0.078$ to 0.268; $P < 0.001$) always was observed between subpopulation pairs from WA and the eastern states (VIC, SA, and NSW). For example, subpopulations MTB-03 and LOC-03 were sampled from the two most distant sites at Mt. Barker, WA and Lockhart, NSW (3,500 km apart), with $F_{ST} = 0.112$ ($P < 0.001$) (Table 4).

Genetic differentiation also was examined over the 2 years of the study, first Australia-wide and second at the Mt. Barker and Struan sites, where samples were collected during consecutive years. There was $<1\%$ genetic differentiation between the Australia-wide subpopulations ($F_{ST} = 0.007$, $P < 0.001$), moderate differentiation at Struan ($F_{ST} = 0.139$, $P < 0.001$), and no genetic differentiation at Mt. Barker ($F_{ST} = 0.003$, $P > 0.05$). Genetic differentiation between populations sampled from disease nurseries and commercial fields differed in the two regions. When subpopulations were grouped on the basis of these parameters and compared across Australia, there was 4% genetic differentiation ($F_{ST} = 0.036$, $P < 0.001$) between nurseries and commercial fields. When compared across regions, WA nurseries and eastern Australian nurseries had 10% genetic differentiation ($F_{ST} = 0.106$, $P < 0.001$), whereas WA nurseries and eastern Australian commercial fields had 12% genetic differentiation ($F_{ST} = 0.116$, $P < 0.001$). Within the region of eastern Australia, there was $<1\%$ genetic differentiation between disease nurseries and commercial fields ($F_{ST} = 0.007$, $P < 0.01$). The genetic differentiation between WA nurseries and eastern Australia subpopulations is more likely to be due to the overall east-west differentiation between the subpopulations than the management of the site from which the isolates were derived.

FCA revealed a geographical pattern of genetic variation in accordance with the results of the AMOVA and F_{ST} analyses, and similarly suggested the distinct genetic composition of eastern (1, 2, 3, 6, 7, 8, 9, 12, and 13) and western (4, 10, and 11) groups of subpopulations (Fig. 2). Subpopulation LA1-02 (Fig. 2, denoted as 5) sat separately from the other subpopulations on the graph. The alleles *MinLm1*-04, *MinLm1*-04, *Lema* 12-03, and *Lema* 12-06, which had a high frequency in the WA subpopulations, contributed most to the construction of the first correspondence axis (highest absolute contribution), and their pattern of variance was best represented by it (highest relative contribution). Additional alleles that also displayed high relative contributions to FCA axis 1 were *Lema* 34-02, *MinLm1*-03, *Lema* 34-03, *Lema* 37-08, and *Lema* 12-08. The expansion and construction of FC2 was associated mostly with alleles that had a high frequency in

TABLE 4. Mean F_{ST} (θ) estimates based upon eight loci for subpopulation pairs of *Leptosphaeria maculans* sampled within Australia^a

Subpopulation	STR-02	TH1-02	TH2-02	MTB-02	LA1-02	STR-03	EDI-03	LA2-03	WON-03	MTB-03	WOH-03	LOC-03	GRE-03
STR-02	...	**	***	***	***	***	**	***	***	***	***	***	***
TH1-02	0.141	...	NS	***	***	NS	**	NS	NS	***	***	NS	NS
TH2-02	0.131	0.017	...	***	***	NS	**	NS	NS	***	***	NS	NS
MTB-02	0.224	0.125	0.126	...	***	***	*	***	**	NS	NS	***	***
LA1-02	0.274	0.280	0.216	0.281	...	***	***	***	***	***	***	***	***
STR-03	0.139	0.008	0.013	0.084	0.263	...	NS	NS	NS	***	***	NS	**
EDI-03	0.107	0.112	0.099	0.097	0.298	0.082	...	NS	NS	*	**	NS	***
LA2-03	0.121	0.006	0.007	0.116	0.237	0.005	0.082	...	NS	***	***	NS	NS
WON-03	0.165	0.028	0.018	0.053	0.215	0.004	0.095	0.016	...	***	***	NS	NS
MTB-03	0.231	0.141	0.147	0.003	0.297	0.104	0.095	0.139	0.078	...	NS	***	***
WOH-03	0.268	0.164	0.187	0.027	0.374	0.116	0.155	0.164	0.112	0.017	...	***	***
LOC-03	0.133	0.019	0.004	0.105	0.213	0.007	0.083	0.001	0.017	0.112	0.144	...	*
GRE-03	0.135	0.031	0.031	0.153	0.313	0.046	0.131	0.036	0.040	0.184	0.191	0.051	...

^a Genetic differentiation determined by θ (42) is below the diagonal, and the probability that allelic distributions are identical between sampling sites when all loci are combined is above the diagonal; * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ indicate nominal significance levels of θ tested by permutation (78,000 times) (11).

subpopulation LA1-02, such as *Lema* 12-07, *Lema* 6-01, and *MinLm1*-06. Altogether, FC axis 1 explained 31% of the variance in the association between alleles and individuals, while FC axes 2 and 3 explained 19 and 12%, respectively.

Bayesian clustering analysis of the multilocus haplotype data set using STRUCTURE strongly supported the existence of three populations of *L. maculans* across Australia (Fig. 3). When three populations ($K = 3$) were assumed on the basis of a high posterior probability ($P[K|X] = 1$), there was a striking difference in the frequencies of isolates assigned probabilistically to population 1 (Fig. 3, gray). This genetic population was from Western Aus-

tralia, with the majority of isolates from the WA subpopulations (MTB-02, MTB-03, and WOH-03) assigned with higher probability to population 1 (Fig. 3, gray). Isolates sampled from eastern Australian subpopulations were most frequently assigned with near equal probability ($\approx 50\%$) to populations 2 and 3 (Fig. 3, black and white, respectively), demonstrating that, within the eastern Australian subpopulations, there is a high degree of population admixture. This admixture means that each individual draws a fraction of its genome from each of the K populations, with mixed ancestry occurring most frequently in the eastern Australian subpopulations. There was no separation between the

Fig. 2. Factorial correspondence analyses (FCA) showing relationships among 13 subpopulations of *Leptosphaeria maculans* in a multivariate space. The axes FC1, FC2, and FC3 are the first, second, and third principal factors of variability, respectively. FC axis 1 explained 31% of the variance in the association between alleles and individuals, while FC axis 2 was 19% and FC axis 3 was 12%. The subpopulations are denoted by numbers, corresponding to the order in which they are listed in Table 1, and all except one fall into the western or eastern group.

Fig. 3. Estimated membership coefficient for each individual isolate of *Leptosphaeria maculans* in each of the $K = 3$ populations as seen by STRUCTURE analysis. Each isolate is represented by a single vertical bar divided into three color segments corresponding to the inferred membership in populations 1 (gray), 2 (black), or 3 (white). Isolates are grouped by subpopulation and arranged sequentially from western (left) to eastern (right) geographical sampling sites. The Western Australian subpopulations contain mostly population 1 isolates, whereas eastern Australian subpopulations are an admixture of populations 2 and 3.

eastern Australian subpopulations upon the basis of origin from disease nurseries or commercial fields, with the admixed populations 2 and 3 occurring across all eastern Australian sites for both nurseries and commercial fields. The STRUCTURE analysis also suggested the presence of some putative recent migrants with admixed isolates of populations 2 and 3 in the WA subpopulations, whereas isolates with predominantly population 1 ancestry were less common among the eastern Australian isolates.

The analysis of logarithmic geographic distances and Rousset's genetic distance of $F_{ST}/(1 - F_{ST})$ (38) over all subpopulations demonstrated a significant correlation between these measures ($r = 0.214$, $P = <0.05$), indicating a significant increase in genetic distance as the distance between populations increased (Fig. 4). When this relationship was examined among subpopulations within a region, there was a significant correlation only among the three WA subpopulations ($r = 0.901$, $P < 0.0001$), in contrast to the subpopulations of eastern Australia ($r = -0.018$, $P > 0.05$). This finding does not support an increase in genetic distance with increasing geographic distance between subpopulations.

DISCUSSION

One of the most significant findings of this study was the moderate degree of genetic differentiation between subpopulations of *L. maculans* from different regions and sites across the Australian continent. This is consistent with a report of genetic differentiation between populations from two fields in Canada (23). No differentiation between populations in Australia had been predicted on the basis of our previous study of Australian subpopulations using restriction fragment length polymorphisms (RFLPs) and AFLPs (4). However, this previous study used only four RFLP markers, and the AFLP markers were either extremely abundant or extremely rare and did not reveal similarities between isolates from the same population. The eight microsatellite and minisatellite loci in the current study were very informative for determining gene diversity and genetic differentiation, with the exception of one locus, *Lema* 39, which is discussed later.

Although *B. napus* is cultivated as a rotation crop in southern Australia, our findings do not support the presence of one widespread population of *L. maculans*. Eastern and Western Australia are separated by the Nullarbor Plain, comprising $\approx 1,200$ km of arid to semi-arid desert country. This natural barrier may restrict gene flow by long-distance dispersal of airborne spores between regions. In the absence of gene flow over large distances, random genetic drift will cause isolated populations to develop different allele frequencies at neutral loci, creating divergence between populations (15). Furthermore, the evidence of genetic differences between subpopulations from the east and west were strengthened when population structure was inferred without regard to the geographical location of the samples using STRUCTURE analysis. Three populations were inferred with the highest probability, with isolates from the three Western Australian subpopulations being assigned most commonly to population 1, whereas isolates from eastern Australia were assigned to the admixed populations 2 and 3. These data indicate that eastern Australian isolates have diverse ancestry and are recombinants of population 2 and 3.

The lack of genetic differentiation between subpopulations from disease nurseries and commercial fields in eastern Australia may be due to the practice of planting nurseries on the stubble of commercial crops at Lake Bolac and Wonwondah. Nonetheless, this finding suggests that the screening practices in eastern Australia are exposing *B. napus* lines to similarly diverse populations of *L. maculans*. A limitation of our study, in terms of understanding the populations in Australian commercial fields, is that such fields were not sampled in WA. Our results contrast to the findings for another plant–fungus interaction, chickpea and *Ascochyta rabiei* in the U.S. Pacific Northwest. The population structure of isolates sampled from a disease nursery was very

different from those sampled from commercial chickpea fields, and this finding led to changes in disease nursery management practices (35).

Another case of east-west genetic differentiation in Australia has been reported for the barley scald pathogen *Rhynchosporium secalis* (27). The genetic structure of a single *R. secalis* population from WA differed significantly from four NSW populations (27). In contrast to the present study, however, the Western Australian population of *R. secalis* had less genotypic diversity than the eastern populations. For both species (*R. secalis* and *L. maculans*), the east-west genetic differentiation may have been due to the presence of a large arid desert between these two regions.

Within regions, genetic differentiation between subpopulation pairs of *L. maculans* usually was related to the geographic distance between sites. Low F_{ST} estimates usually were associated with subpopulation pairs from the same state or different states in eastern Australia. An isolation by distance effect among *L. maculans* subpopulations in Australia only existed between the subpopulations from Western Australia, which were few in number. Frequent gene flow between subpopulations may have reduced genetic differentiation by homogenizing alleles among subpopulations. Conidia of *L. maculans* account for only localized spread between plants within a field, because they are spread via splash dispersal. Ascospores are windborne and can disperse 8 to 10 km (6), with most released within a short range of the previous year's crop. Other factors that influence ascospore dispersal are wind speed and direction (24). The large area grown to *B. napus* in Australia and continuous cropping through rotation ensures the persistence of inoculum and, consequently, of blackleg disease (21,24). Although few ascospores may travel tens of kilometers, only a very low rate of migration per generation is required to arrest gene fixation.

A second explanation for low genetic differentiation is gene flow by the movement of infected plant material. The low genetic differentiation between the Mt. Barker and Wongan Hills subpopulations, which are separated by 425 km, may have been due to the past practice of moving *B. napus* stubble from the Mt. Barker nursery to the Wongan Hills district in order to increase disease pressure in the blackleg nursery (Graham Walton, Department of Agriculture Western Australia, *personal communication*). Seed transmission, as discussed above, provides another method of gene flow via infected plant material. Commercial seed are now fungicide treated and internal infection by *L. maculans* has not been observed (4). However, seed transmission cannot be

Fig. 4. Isolation–distance relationship between all subpopulation pairs of *Leptosphaeria maculans*, where $F_{ST}/(1 - F_{ST})$ was regressed over distances between sampling sites in Australia. The correlation (r) between genetic distance $F_{ST}/(1 - F_{ST})$ and log geographic distance (km) was significant, as assessed by a Mantel test between all subpopulation pairs ($r = 0.214$, $P < 0.05$).

excluded as a mechanism of past gene flow because little information exists on bulk seed transportation, especially during the establishment of the industry, and many farmers retain seed from their own crops in preference to buying commercial seed.

The finding of high genotypic diversity within Australian subpopulations of *L. maculans* concurs with results from previous studies where a high level of genotypic diversity was found regardless of whether ascospores (16,23) or conidia (4,5,16) were sampled. Given the high genotypic diversity and even distribution of mating types reported in Australia (4,5,16), the null hypothesis of random mating was expected to be supported in the current study. The rejection of this hypothesis for seven clone-corrected subpopulations was unexpected. It was caused by linkage between the microsatellite locus *Lema* 39 and other loci in six of the seven subpopulations. Subsequent sequence analysis revealed that *Lema* 39 was <1 kb from *MinLm1* and present on two of the *L. maculans* BAC clones from which oligonucleotide primers were designed, including the one from which the minisatellite *MinLm1* was identified. Subsequent crossing studies with several of the other loci have shown that all tested (except *Lema* 39) are unlinked and segregate independently (data not shown). Although only 4 of the 13 clone-corrected subpopulations displayed gametic disequilibrium between locus-pair *Lema* 39 and *MinLm1*, only one of these two markers should be used in future population genetic studies.

Another finding of this study was that, overall, the Australian *L. maculans* population was genetically stable over the 2 years of the study. At the two disease nursery sites examined on consecutive years, the Mt. Barker subpopulations exhibited no genetic differentiation whereas the Struan subpopulations exhibited moderate genetic differentiation. Nursery management practices may explain the difference. As mentioned previously, the Mt. Barker nursery is sown into stubble of a polygenic cultivar in different sites, whereas the Struan nursery is sown back into the same field which contains stubble of a mixture of cultivars with either polygenic or sylvestris-derived major gene resistance. We have tested the pathogenicity of many isolates described in this article and documented an increase in the number of isolates able to render sylvestris-derived resistance ineffective during the 2 years at Struan (40,41). The repeated selection at the Struan site for isolates virulent on sylvestris-derived cultivars, combined with sexual recombination in *L. maculans*, has resulted in an increase in the frequency of these isolates. Although our study has produced inconclusive results on temporal variation in *L. maculans*, it has indicated that the different practices at Australian blackleg disease nurseries may affect the genetic structure and pathogenicity of *L. maculans*. In the future, *B. napus* breeders and plant pathologists may need to standardize management practices across the country, particularly because these nurseries are used for screening breeding lines and for determining disease resistance ratings of released cultivar.

Control of blackleg disease of *B. napus* is dependent largely upon sowing resistant cultivars, which relies on robust screening methods to assess new sources of resistance that then can be incorporated into adapted lines. Our research has shown that the two disease nurseries in Western Australia tested differed from nurseries and commercial fields in eastern Australia by hosting isolates belonging to one population while eastern Australian isolates were an admixture of two other populations. Genetically subdivided populations could be managed by plant breeders as separate entities, exploiting the various levels of gene flow (or lack of it) as a disease management tool (30). With regards to cultivar deployment, a regional approach to rotations could be adopted because virulent mutants that increase in frequency in populations and overcome sources of resistance conferred by major genes over time will be unlikely to emigrate between the eastern and western regions. Rotation of major resistance genes through time will produce disruptive selection, which may

prevent isolates virulent on a particular cultivar from accumulating to high frequencies in populations (25). Exploitation of quantitative (polygenic) resistance is likely to be more effective in the long term than major gene resistance, given that Australian *L. maculans* populations already contain many combinations of avirulence and virulence alleles that are complementary to major genes (3). It is also important that the system of screening upon which the Australian blackleg resistance rating system is based continues to be performed in all *B. napus*-growing regions at a large number of sites, thus ensuring that breeding lines and cultivars are exposed to a diverse array of genetic and pathogenic populations of *L. maculans*.

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