Genetic Structure of *Rhynchosporium secalis* in Australia

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Accepted for publication 11 May 1999.

ABSTRACT

McDonald, B. A., Zhan, J., and Burdon, J. J. 1999. Genetic structure of *Rhynchosporium secalis* in Australia. *Phytopathology* 89:639-645.

Restriction fragment length polymorphism (RFLP) markers were used to determine the genetic structure of Australian field populations of the barley scald pathogen *Rhynchosporium secalis*. Fungal isolates were collected by hierarchical sampling from five naturally infected barley fields in different geographic locations during a single growing season. Genetic variation was high in Australian *R. secalis* populations. Among the 265 fungal isolates analyzed, 214 distinct genotypes were identified. Average genotype diversity within a field population was 65% of its theoretical maximum. Nei's average gene diversity across seven RFLP loci was 0.54. The

majority (76%) of gene diversity was distributed within sampling site areas measuring $\approx 1 \text{ m}^2$; 19% of gene diversity was distributed among sampling sites within fields; and 5% of gene diversity was distributed among fields. Fungal populations from different locations differed significantly both in allele frequencies and genotype diversities. The degree of genetic differentiation was significantly correlated with geographic distance between populations. Our results suggest that the *R. secalis* population in Western Australia has a different genetic structure than populations in Victoria and South Australia.

Additional keywords: gene flow, genetic diversity, hierarchical diversity analysis.

Knowledge of the amount and distribution of genetic variation within and among populations is an important component of understanding the population biology of pathogenic fungi. Genetic structure can be used to infer the relative impact of different forces that influence the evolution of pathogen populations. In turn, a better understanding of evolutionary forces may allow us to predict the potential for pathogen populations to evolve in agricultural ecosystems (43). For example, a large amount of genetic diversity distributed over a small spatial scale suggests the possibility of rapid adaptation by a pathogen to a changing environment (e.g., new host resistance genes or fungicides). A high degree of genetic similarity among populations collected from widely separated geographic regions suggests the occurrence of significant long-distance dispersal and gene flow. Gene flow over long distances poses a threat to deployment of resistance genes tailored to local pathogen populations, because immigrants may possess virulence genes that can overcome resistance in local host cultivars. New virulence genes also might be incorporated into local pathogen populations through recombination (6).

The fungus *Rhynchosporium secalis* (Oudem.) J.J. Davis causes barley scald disease. *R. secalis* can be a significant pathogen anywhere barley crops are grown (42), causing 10 to 40% reductions in yield under favorable conditions (1,23,37). It is thought that the primary inoculum of *R. secalis* consists of conidia dispersed by rainsplash or mycelia in infected seeds (3,40). The teleomorph of *R. secalis* has not yet been identified.

Although the number of pathotypes identified in different parts of the world varies substantially (from 2 to 75 pathotypes have been identified in previous studies [2,9,12,13,18,43]), it appears that genetic variation in *R. secalis* populations is generally high

(7,8,20,25). Isolates secured from different scald lesions taken from the same plant and even different spores isolated from the same lesion may vary significantly for sporulation rate, virulence, and isozymes (7,15,32). Furthermore, the virulence structure of populations of *R. secalis* may change significantly over a relatively short period of time (50). Goodwin et al. (13,14) used isozyme markers to study the genetic structure of *R. secalis* populations originating in different geographic regions. They found that a large proportion of the total genetic diversity was distributed within local populations.

Major resistance genes have been deployed in barley to control scald; however, these genes have frequently exhibited a finite life span due to the breakdown of resistance associated with selection for increased virulence in *R. secalis* populations (16,49). To more effectively use resistance genes in plant-breeding programs, knowledge of the population biology of *R. secalis* would be useful (46). The principal objective of the current study was to use restriction fragment length polymorphism (RFLP) markers and hierarchical sampling to determine the genetic structure of *R. secalis* populations in Australia.

MATERIALS AND METHODS

Field sampling methods. Barley leaves infected with *R. secalis* were sampled from five locations in Australia during October and November of the 1996 barley-growing season. All leaf samples were collected in naturally infected fields near the end of the growing season. Each leaf was collected from a different plant or tiller. Collection 1 was a fine-scale transect sampling from the Wagga Wagga Agricultural Institute, New South Wales. Four parallel, 6-m transects separated by 18 cm were laid out in a plot sown to cv. Clipper barley. One infected leaf was collected every 30 cm along each transect (20 leaves per transect; Fig. 1). The remaining four collections were taken from farmer's fields by a larger scale hierarchical sampling strategy (Fig. 1). Collection 2 was from cv. O'Connor grown at Methul, New South Wales; col-

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lection 3 was from cv. Schooner grown at Rannock, New South Wales; collection 4 was from cv. Arapiles grown at Horsham, Victoria; and collection 5 was from cv. Stirling grown at Quindanning, Western Australia. For each field population, three sites were sampled along two parallel transects. Both transects and collection sites along transects were separated by 10 m. Infected leaves (10 total) were sampled from each site within the field (60 leaves per field total). A total of 320 infected leaves were collected from the five locations.

Isolation and growth of *R. secalis* strains. Leaves were placed in paper envelopes and allowed to air-dry at room temperature for at least 48 h. Dried leaves were wet for 10 s in 70% ethanol, surface-sterilized for 60 s in a 0.5% sodium hypochlorite solution, and rinsed in distilled water for 10 s. Wet leaves were pressed dry between paper towels and placed on a plastic screen that rested on rubber bands above damp filter paper in a petri dish. Petri dishes were incubated in the dark at 15°C for 48 h to induce fungal growth and sporulation. Fine glass needles were used to remove small tufts of mycelia that emerged around lesion borders. In 58 cases, multiple lesions were sampled from the same leaf. In eight cases, more than one isolation was made from the same lesion. Mycelial tufts were transferred to petri dishes containing lima bean agar (LBA). After incubating at 15°C for 1 to 2 weeks, colonies were

spread across fresh LBA plates and incubated for an additional 2 to 3 weeks at 15°C. Spores and mycelial fragments were harvested by adding 1 ml of sterile distilled water and scraping gently across the mycelium with a sterile glass microscope slide. The resulting slurry of spores and mycelium was used to inoculate 40-ml flasks containing potato dextrose broth amended with kanamycin at 50 µg/ml and to inoculate 2.0-ml cryovials (two-thirds full) containing silica gel for long-term storage. Inoculated flasks were incubated on a shaker at 15°C for 3 to 4 weeks.

DNA extraction. Mycelium was harvested by centrifugation in a 15-ml polypropylene centrifuge tube and lyophilized for 48 h.

Fig. 1. Collection strategies used to sample field populations of *Rhynchosporium secalis* in Australia. **A**, Locations of five field populations sampled. **B**, Six-site ordered hierarchy (not drawn to scale). Ten infected leaves were sampled at regular intervals in a circular sweep from different plants or tillers at each field site. Three field sites were spaced at 10-m intervals along two parallel transects separated by 10 m. Sixty leaves total were sampled per field. **C**, High-density ordered transects (not drawn to scale). Infected leaves were collected at 30-cm intervals over a 6-m transect. Four parallel transects were spaced 18 cm apart. Eighty leaves total were sampled per field.

Fig. 2. Example restriction fragment length polymorphism alleles in 12 Australian isolates of *Rhynchosporium secalis* originating from one site in the Methul field population. λ -HindIII size standards are shown in the final lane to the right. **A**, Probe pRS26 produced a repetitive hybridization profile useful for DNA fingerprinting; **B**, probe pRS6; **C**, probe pRS49; and **D**, probe pRS2.

Lyophilized tissue was crushed to a fine powder with a glass rod. Warmed (42°C) extraction buffer (5 ml of 1% sodium lauroyl sarcosine, 150 mM EDTA, 50 mM Tris pH 8.0, and proteinase K [P-8044, Sigma-RBI, Natick, MA] at 300 mg/liter) was added to each tube and mixed with the powdered fungal tissue in a vortexer for 30 to 45 s. Tubes were placed in a 42°C water bath for 10 to 30 min. Phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol; 5 ml) was added, and tubes were vortexed to form an emulsion. Tubes were centrifuged at $28,000 \times g$ for 10 min to separate phases. The aqueous phase was transferred to a fresh 15-ml tube and extracted with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol). Phases were separated at $28,000 \times g$ for 10 min. The aqueous phase was transferred to a fresh 15-ml tube, and DNA was precipitated by mixing in 2 volumes of 95% EtOH and 0.5 volumes of 7.5 M ammonium acetate and left to stand at room temperature for 20 min. DNA pellets were formed by centrifugation at $2,600 \times g$ for 10 min at room temperature. DNA pellets were dissolved in 400 μ l of Tris-EDTA buffer and transferred to 1.5-ml microfuge tubes. DNA concentrations were determined with a fluorometer (DyNA Quant 200, Hoefer Pharmacia Biotech Inc., San Francisco).

Development of probes for RFLP analysis. A library of anonymous, random DNA fragments was constructed for use as probes. Total DNA from a randomly selected isolate of *R. secalis* collected in 1985 (RS14 [26]) was digested with *Bam*HI. The resulting fragments were ligated into pUC18 and cloned in *Escherichia coli* by standard methods (24). Approximately 100 clones were picked for use as probes in RFLP analysis. Thirty-five randomly chosen clones were hybridized to DNA digested with *Pst*I, *Eco*RI, *Hind*III, and *Bam*HI from six Californian strains that had different isozyme phenotypes (26). On the basis of the preliminary screen, *Eco*RI was chosen as the most polymorphic enzyme, and eight probes were chosen for RFLP analysis based on strong, easily interpreted hybridization patterns. The eight probes were hybridized to DNA from 52 *R. secalis* isolates from California that had been characterized previously for four isozyme loci (26). One of the probes (pRS26) hybridized to several well-separated fragments in each isolate, suggesting it might be useful for DNA fingerprinting.

Data analysis. For the seven probes that hybridized to single loci, each probe-enzyme combination was treated as a different RFLP locus. DNA fragments or combinations of fragments with different sizes were treated as alleles at each RFLP locus. Only alleles that could be scored unambiguously were included in the analysis. Multi-locus haplotypes for each isolate were formed by joining the alleles present at each locus in the same order to form a seven-digit number that described the haplotype for each isolate. For the pRS26 probe, each hybridizing fragment profile was treated as a DNA fingerprint. Isolates with the same DNA fingerprint and multilocus haplotype were considered individual members of the same clone.

Genetic variation in each population was quantified by measures of gene (30) and genotype (41) diversity. Total gene diversity was partitioned into components among field populations, among sites within field populations, and within sites within field populations by hierarchical gene diversity analysis (5,30). All hierarchical gene diversities were estimated by weighted gene frequencies for small and unequal sample sizes (30,31). Only the collections from Methul, Rannock, and Horsham were included in the hierarchical analysis. The Wagga Wagga collection was excluded because a nonhierarchical sampling method was used; the Quindanning (Western Australia) population was excluded because the genetic structure of the population was characterized by a higher degree of clonality that was significantly different from the other populations.

Similarities in the genetic structure of populations were evaluated based on contingency χ^2 tests for differences in allele frequencies (45), Nei's (29) measure of genetic distance, and a Student's *t* test for differences in genotype diversities (10). For the contingency χ^2 test, alleles present at frequencies lower than 0.05 in all five populations were pooled into a single category. The χ^2 value obtained by Workman and Niswander's method (45) was

divided by two because *R. secalis* is a haploid organism. The results from the χ^2 and *t* tests were corrected by the Bonferroni procedure as proposed by Weir (44).

A test for isolation by distance was based on the method described by Slatkin (39). Gene flow was estimated based on the average G_{ST} (population differentiation) across seven RFLP loci (30), using the formula $Nm = (1/G_{ST} - 1)/2$, where Nm is the average number of migrants exchanged between populations in each generation. If the degree of gene flow among populations is affected by geographic distance, we would expect to find a significant negative correlation between the logarithm of Nm and the logarithm of distance between populations (39).

RESULTS

RFLP probes. Of the 146 probe-enzyme combinations tested, 51 were polymorphic among the California isolates used in the screen for RFLPs. Of the 20 probes that appeared useful for RFLP analysis with *Eco*RI, 8 were used to characterize the Australian collections. Seven of the selected probes showed simple hybridization patterns consistent with a single genetic locus (Fig. 2). The eighth probe, pRS26, generated a complex hybridization pattern with the majority of California and Australian isolates assayed (Fig. 2). Although we did not validate the use of pRS26 for DNA fingerprinting in the Australian collections, in the California collection pRS26 hybridized to an average of 8.9 fragments between 2.0 and 9.4 kb in size in each isolate. For 36 scored fragments, the average frequency was 0.245 among the 52 California isolates. Using the method of Jeffrey et al. (19), we estimated that the average probability that two isolates chosen at random would have the same hybridization profile was 4×10^{-6} , suggesting pRS26 might be useful for DNA fingerprinting in other populations, including Australia.

Genetic variation in Australian *R. secalis* populations. A total of 265 isolates were included in the analysis of 5 Australian collections. The number of alleles found at 7 RFLP loci ranged from 2 to 5, with an average of 3.1 alleles per locus. For all RFLP loci, the summed frequency of the two most common alleles was at least 70% (Table 1). Significant levels of gene and genotype diversity

TABLE 1. Allele frequencies at seven restriction fragment length polymorphism (RFLP) loci in Australian field populations of *Rhynchosporium secalis* collected from the Wagga Wagga Agricultural Institute (WWAI), Methul, Rannock (Rann), Horsham (Horsh), and Quindanning (Quindan) during the 1996 barley-growing season

Loci	Allele ^a	WWAI	Methul	Rann	Horsh	Quindan
RS37	1	0.68	0.75	0.82	0.56	0.68
	3	0.32	0.25	0.18	0.44	0.05
	4	0.00	0.00	0.00	0.00	0.28
RS35	1	0.42	0.45	0.57	0.65	0.26
	2	0.42	0.46	0.33	0.12	0.26
	3	0.00	0.05	0.05	0.00	0.44
	10	0.10	0.00	0.02	0.18	0.00
RS6	3	0.00	0.09	0.00	0.00	0.29
	4	0.61	0.38	0.51	0.15	0.31
	6	0.16	0.33	0.16	0.21	0.02
RS47	8	0.23	0.16	0.33	0.62	0.38
	1	0.47	0.56	0.49	0.54	0.33
	2	0.21	0.07	0.11	0.00	0.14
RS52	3	0.32	0.37	0.40	0.46	0.52
	1	0.07	0.11	0.07	0.05	0.26
	2	0.62	0.65	0.62	0.62	0.74
RS36	3	0.31	0.24	0.32 ^b	0.33	0.00
	1	0.00	0.05	0.08	0.03	0.00
	2	0.53	0.36	0.25	0.45	0.52
RS2	3	0.37	0.54	0.67	0.53 ^b	0.48
	5	0.08	0.05	0.00	0.00	0.00
	1	0.47	0.21	0.59	0.68	0.37
RS2	2	0.53	0.79	0.41	0.32	0.63

^a Alleles present at frequencies lower than 0.05 across all five populations are not shown.

^b Summed frequencies are greater than 1.0 due to rounding error.

were found in the Australian populations (Table 2). Nei's (30) measure of gene diversity averaged across the seven loci ranged from 0.50 to 0.55 within populations. Stoddart and Taylor's (41) measure of genotype diversity ranged from 45 to 97% of its theoretical maximum (equal to sample size) across the five populations (Table 2). The collection from Rannock had the highest genotype diversity, with 60 genotypes identified among 61 isolates.

Distribution of genetic diversity within and among populations. The majority of gene and genotype diversity was distributed on a small spatial scale. Hierarchical gene diversity analysis indicated that ~5% of total gene diversity was distributed among field populations, 18% among sampling sites within a field, and 76% within sites within a field. Genotype diversity also was distributed on a fine scale, except in the West Australia (Quindanning) field population. In Methul, Rannock, and Horsham, an average of 7.8 genotypes were found among 8.7 isolates sampled from each site within each field. Isolates with the same DNA fingerprints and multilocus haplotypes were found in the same field site, with only one case (in Horsham) in which the same clone was shared between different sites within a field. In contrast, the Quindanning population had an average of 4.1 genotypes among 7.0 isolates sampled from each field site, and 3 identical genotypes were found at two or three sites within this field. There were 13 cases in which the same multilocus haplotype was found in different field populations. DNA fingerprints showed that isolates having the same multilocus haplotype were genetically different in 10 of the cases. In the other three cases, the DNA fingerprints were similar, but could not be identified with certainty because the number of hybridizing fragments was small, with only three to four hybridizing fragments per case.

Genetic diversity was detected at the smallest spatial scale sampled. In five of eight cases, different genotypes were found among isolates drawn from different parts of the same lesions. For the 58 cases in which different lesions were sampled from the same leaf, different genotypes were found 45 times.

Population differentiation and isolation by distance. Significant differences in allele frequencies existed among populations

for all RFLP loci (Table 3). The level of population differentiation ranged from 0.03 to 0.11 across the seven RFLP loci, with an average of 0.07. Pairwise comparisons of genotype diversity and genetic distance are shown in Table 4. Significant differences in genotype diversity were found in 6 of 10 comparisons. Further analysis by the Bonferroni procedure corroborated the significance of differences across all populations at $P < 0.01$. The correlation coefficient between degree of gene flow and geographic distance was -0.79 (Fig. 3), which was significant at $P < 0.05$, indicating a significant decrease in Nm as distance between populations increased.

DISCUSSION

The goal of our study was to use RFLP markers to characterize the genetic structure of *R. secalis* populations in Australia. Unlike plants and animals, fungi have few morphological characters that can be used to study their population biology. DNA markers, however, have provided a powerful tool for this type of study. Over the last decade, DNA markers have been used to quantify genetic variation (4,38), decipher spatial distribution (27), and distinguish among forces that generate and maintain genetic variation (47,48) for several agriculturally important fungi. Our study is the first to use anonymous RFLP markers to examine the genetic structure of *R. secalis* populations. Our results showed that Australian populations of *R. secalis* were highly variable and that the great majority of genetic variation was distributed within fields on a fine spatial scale. Collections made from different locations in southern Australia showed a moderate to high degree of genetic similarity. However, it appears that the Western Australia (Quindanning) population is genetically distinct from the other sampled populations.

Because *R. secalis* is thought to reproduce exclusively asexually, it was expected that populations of this fungus would display low genotype diversity among a limited number of clonal lineages. Our observations did not support this hypothesis, however. Many distinct genotypes were recovered from each field population over relatively small geographic areas. In the Methul field population, 60 genotypes could be differentiated among 61 isolates analyzed. High genetic diversity and relatively low levels of clonality (except for Quindanning) indicated that the field populations of *R. secalis* originated from genetically diverse source populations. The effective population size of *R. secalis* in Australian barley fields appears sufficiently large to limit the effects of genetic drift caused by seasonal epidemic cycles.

Hierarchical analysis (5,30) enabled us to partition the total genetic variation among several spatial scales for the three populations from southern Australia (Horsham, Methul, and Rannock) included in this analysis. The geographic distances among these populations ranged from 15 to 500 km. We partitioned total gene diversity into three components. The majority of gene diversity (76%) was distributed within field sampling units covering an area of ~1 m². Only 5% of gene diversity was distributed among field populations in our survey; a finding that is consistent with a previous study showing

TABLE 2. Sample sizes, average gene diversities (Nei and Chesser [31]) and indicators of genotype diversity for five Australian *Rhynchosporium secalis* field populations sampled in 1996

Population	Sample size	Gene diversity	No. of genotypes ^a	Percentage of maximum ^b
Wagga Wagga	66	0.548	49	48
Methul	57	0.517	51	80
Rannock	61	0.505	60	97
Horsham	39	0.506	29	55
Quindanning	42	0.538	25	45

^a Based on combination of DNA fingerprints and multilocus haplotypes.
^b Genotype diversity expressed as percentage of maximum possible value of Stoddart and Taylor (43).

TABLE 3. Contingency χ^2 test for differences in allele frequencies and G_{ST} (population differentiation) across five field populations of *Rhynchosporium secalis* from Australia

RFLP ^a loci	χ^2 Test		Pairwise comparisons of population subdivision (G_{ST}) ^b										
	Value ^c	df	C1-C2	C1-C3	C1-C4	C1-C5	C2-C3	C2-C4	C2-C5	C3-C4	C3-C5	C4-C5	Overall
RS37	77.2***	8	0.006	0.024	0.013	0.068	0.006	0.040	0.066	0.077	0.070	0.110	0.071
RS35	93.3***	16	0.037	0.010	0.009	0.109	0.014	0.074	0.081	0.033	0.091	0.139	0.084
RS6	45.3***	16	0.124	0.104	0.327	0.212	0.030	0.097	0.063	0.079	0.052	0.073	0.095
RS47	16.1*	8	0.014	0.010	0.028	0.028	0.003	0.006	0.034	0.007	0.017	0.030	0.025
RS52	26.9***	8	0.027	0.019	0.018	0.090	0.004	0.006	0.046	0.000	0.074	0.087	0.040
RS36	29.7**	12	0.005	0.007	0.015	0.032	0.016	0.005	0.016	0.030	0.057	0.004	0.047
RS2	22.9***	4	0.023	0.055	0.083	0.001	0.149	0.218	0.031	0.008	0.049	0.094	0.109

^a Restriction fragment length polymorphism.
^b C1, C2, C3, C4, and C5 are population samples collected from Wagga Wagga, Methul, Rannock, Horsham, and Quindanning, respectively.
^c *, **, and *** indicate significance at $P = 0.05$, 0.01 , and 0.001 , respectively.

that the majority of isozyme genetic variation was distributed within regions (13). Our results suggest that *R. secalis* propagules are effectively disseminated over spatial scales of at least hundreds of kilometers, leading to only minor differences in the distribution of genetic diversity among field populations on a regional scale. Conidia dispersed by rainsplash are not expected to travel long distances, so it is unlikely that these populations are linked by movement of conidia. It is more likely that gene flow among these populations occurs through infected seeds, fungal spores (possibly ascospores from the unknown teleomorph), or infected plant material in hay, which can be disseminated over longer distances.

The fact that 95% of the total RFLP variation was distributed within fields suggests that *R. secalis* populations have the potential to evolve relatively quickly. High diversity in neutral RFLP markers does not mean that diversity in phenotypic characters, such as virulence, also will be high. We did not test isolate virulence, however. It is possible that diversity for virulence is much lower than diversity for RFLPs, especially if there is strong selection due to scald-resistance genes present in a barley population. One advantage of neutral genetic markers is that they allow determination of pathogen genetic structure independent of selection. In this case, our data showed that Australian field populations of *R. secalis* are composed of many genotypes distributed on a small spatial scale. We believe that this genetic structure offers greater evolutionary potential than a clonal genetic structure (such as *Puccinia graminis* f. sp. *tritici* in Australia or the Great Plains of the United States), in which few genotypes are widely distributed. We hypothesize that the diverse *R. secalis* populations in Australia could adapt rapidly to newly introduced resistance genes, and therefore, the traditional method of introducing single major resistance genes into good agronomic cultivars may not provide long-term control of barley scald disease (13).

The larger pairwise genetic distances (Table 4) and lower genotype diversity found in the Quindanning population compared with other field populations suggest that the *R. secalis* population originating from Western Australia differed significantly from the other populations. We have considered four possible explanations for the genetic differentiation between the two regions. The first possibility is that adaptation to the local environment, including barley cultivars with different resistance genes, has led to genetic differentiation. Selection for virulence genes or linked genes (hitchhiking) that overcome resistance in local cultivars could lead to adaptation to local environments and promote genetic divergence of populations. In our case, selection would need to operate on anonymous RFLP markers or linked loci (28) to cause population differentiation. This possibility is considered unlikely because the probes used were selected from a random sample of fragments of total genomic DNA.

A second possibility is population subdivision due to limited gene flow between Western Australia and other populations. Restricted

gene flow among geographically isolated populations allows random fixation of different neutral alleles as a result of genetic drift. Under a model in which gene flow decreases with increasing distance, organisms exhibiting restricted dispersal are expected to show genetic isolation by distance (28,39). The isolation by distance model is supported by our results. There was a significant negative correlation between the logarithm of Nm and the logarithm of distance between populations. In Australia, there can be significant movement of farm products (especially straw used for animal feed) between east and west as a result of regional droughts. This could offer a mechanism for periodic gene flow over long distances.

A third possibility is that the Western Australia population had a different origin or was introduced at a different time than the other populations. We do not know when *R. secalis* was introduced into Australia or whether there were several independent introductions. Comparisons with other populations around the world may permit testing of this hypothesis. A fourth possibility is that the Quindanning field population is not representative of Western Australia and that the apparent differences between regions are an artifact of limited sampling. This possibility can be tested with additional collections from Western Australia.

The amount of genetic variation measured in our study was higher than the variation found in previous studies based on isozymes. Goodwin et al. (14) found that the isozyme gene diversity in *R. secalis* populations collected from Australia ranged from 0.123 to 0.169. It is likely that RFLP markers are more sensitive and offer higher resolution than isozyme markers. During the development of RFLP markers, we used a California collection of *R. secalis* that had been characterized previously with isozymes (26). The most common isozyme multilocus haplotype (based on four isozyme loci) in the California field population was found 11 times in our reference sample of 52 isolates. The isozyme haplotype was resolved into three distinct RFLP haplotypes based on four RFLP loci. Seven DNA fingerprints were found among the eleven strains with the repetitive probe pRS26. The increased genetic diversity found also could be caused by the larger number of isolates evaluated in our sample. Our sample size was large enough to detect low-frequency alleles that have the potential to increase the overall measure of gene diversity. Although there was no evidence of significant population differentiation based on G_{ST} , the collections from southern Australia had significant differences in allele frequency for several loci (results not shown), indicating the sample sizes in our study were large enough to detect minor differences among populations.

The amount and distribution of genetic diversity found in field populations of *R. secalis* were similar to those found in *Mycosphaerella graminicola* (11) and *Phaeosphaeria nodorum* (22). Field populations of *M. graminicola* and *P. nodorum* are characterized

TABLE 4. Pairwise comparisons of genotype diversity (Stoddart and Taylor [43], above diagonal) and genetic distances (Nei and Chesser [30], below diagonal) among populations collected from different geographic locations in Australia

Population	Wagga Wagga	Methul	Rannock	Horsham	Quindanning
Wagga Wagga	...	2.013 ^a (111) ^b	3.513 ^{***} (121)	0.424 (99)	0.200 (102)
Methul	0.067	...	1.630 (116)	1.794 [*] (94)	3.085 ^{**} (97)
Rannock	0.059	0.069	...	3.639 ^{***} (98)	6.383 ^{***} (101)
Horsham	0.233	0.179	0.083	...	0.803 (79)
Quindanning	0.240	0.155	0.161	0.237	...

^a Student's *t* test values. *, **, and *** indicate significance at $P = 0.05$, 0.01, and 0.001, respectively.
^b Degrees of freedom.

Fig. 3. Correlation between the logarithm of estimated number of migrants per generation (Nm) between each pair of populations and the logarithm of geographic distance for *Rhynchosporium secalis* populations collected from different locations in Australia. Nm was estimated using average G_{ST} (population differentiation) across seven restriction fragment length polymorphism loci (30).

by a high degree of sexual reproduction (11,21,47,48). Airborne ascospores serve as the primary inoculum to establish field populations of *M. graminicola* (33–36), and we have proposed that ascospores also are the primary inoculum for *P. nodorum* (21, 22). After establishment, populations of these fungi expand through a mixture of sexual and asexual reproduction (17,21,22, 36,47,48). The sexual stage has not been reported for *R. secalis*, but a recent study with isozyme markers (8) showed that most of the alleles at isozyme loci were in gametic equilibrium. We have obtained similar results with larger sample sizes based on RFLP data (B. A. McDonald, J. Zhan, and J. J. Burdon, unpublished data). It is possible that the sexual stage of *R. secalis* occurs on stubble between growing seasons and plays some role in the establishment of a founder population, followed by exclusively asexual propagation. We plan to test this hypothesis by monitoring changes in the genetic structure of field populations over the growing season.

ACKNOWLEDGMENTS

Undergraduate students L. Setchfield, P. Bushong, and F. Garcia made the *R. secalis* DNA library, screened the library to find polymorphic probes, and collected the first California data set. The students were supported by National Science Foundation Grant DEB-9306377. R. Pettway supervised the students with support from the Texas Agricultural Experiment Station. B. Read assisted with all collections made near the Wagga Wagga Agricultural Institute. D. Moody and R. Loughman made field collections near Horsham and Perth, respectively. R. Heywood provided technical assistance. B. A. McDonald thanks the Australian Grains Research and Development Council for the support provided by a Visiting Fellowship (VF22).

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