Global Hierarchical Gene Diversity Analysis Suggests the Fertile Crescent Is Not the Center of Origin of the Barley Scald Pathogen *Rhynchosporium secalis*

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ABSTRACT

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A total of 1,366 *Rhynchosporium secalis* isolates causing scald on barley, rye, and wild barley (*Hordeum spontaneum*) were assayed for restriction fragment length polymorphism loci, DNA fingerprints, and mating type, to characterize global genetic structure. The isolates originated from 31 field populations on five continents. Hierarchical analysis revealed that more than 70% of the total genetic variation within regions was distributed within a barley field. At the global level, only 58% of the total genetic variation was distributed within fields, while 11% was dis-

tributed among fields within regions, and 31% was distributed among regions. A significant correlation was found between genetic and geographic distance. These findings suggest that gene flow is common at the local level while it is low between regions on the same continent, and rare between continents. Analyses of multilocus associations, genotype diversity, and mating type frequencies indicate that sexual recombination is occurring in most of the populations. We found the highest allele richness in Scandinavia followed by Switzerland. This suggests that *R. secalis* may not have originated at the center of origin of barley, the Fertile Crescent, nor in a secondary center of diversity of barley, Ethiopia.

Additional keywords: coevolution, host specialization, population genetics, speciation.

Rhynchosporium secalis (Oudem.) J.J. Davis is a haploid fungus causing the scald disease on barley, rye, triticale, and various other members of the family *Poaceae* (8,52). Its worldwide occurrence and economic importance in barley production motivated several studies on the population genetic structure of this pathogen. Neutral molecular markers such as restriction fragment length polymorphisms (RFLPs), isozymes, and random amplified polymorphic DNA (RAPDs) have been used to measure variability in this fungus (7,14,30,32,38–40). These studies found no correlation between markers and pathogenicity or races, but all detected a high degree of genotypic diversity over relatively small spatial scales. For instance, $\approx 75\%$ of the total genetic diversity on a continent was concentrated within a sampling plot of 1 m² (40). This was unexpected for a fungus that was thought to be exclusively asexual and dispersed primarily by rain-splash.

The mechanisms that generate variability in *R. secalis* are still not clear, but hypotheses have included mutation and migration (16), asexual recombination (36,37), and a cryptic sexual stage (32,40). Tests for multilocus associations consistently showed gametic equilibrium (40). Identification of the mating type loci in *R. secalis* (27) made it possible to measure the frequency distribution of mating type alleles in *R. secalis* populations (27). Equal frequencies of opposite mating types consistent with frequency-dependent selection due to random mating were found in 20 of 24 field populations originating from seven countries on four continents.

Little evidence was found for population subdivision over spatial scales of hundreds of kilometers (32,40). Because asexual

conidia are splash-dispersed and do not have the ability for long-distance movement, the potential to overcome differentiation among regional populations was attributed to putative wind-dispersed ascospores (32,40). McDonald and Linde (31) placed *R. secalis* into a relatively high-risk category for evolutionary potential because of its putative mixed reproduction system and moderate dispersal of asexual propagules.

The center of origin of a pathogen is generally also its center of genetic diversity. If *R. secalis* originated as a pathogen of barley, a high genetic diversity would be expected in the Fertile Crescent of the Middle East, which is the center of origin of barley (20,55). Although studies on the genetic variation of *R. secalis* from several continents (7,14,30,32,38–40) including the Middle East (49) have been conducted, different molecular markers were used for most of the studies, making comparisons difficult. Additionally, different sampling methods and population sizes used in each of the studies make it difficult to infer the center of origin of *R. secalis*.

In this study, we analyzed populations of *R. secalis* from Africa, Australia, Europe, the Middle East, and North America. Our aims were to (i) determine the hierarchical genetic structure of *R. secalis* populations over spatial scales ranging from square meters to continents, and (ii) test the null hypothesis that the Middle East is the center of origin for *R. secalis*.

MATERIALS AND METHODS

Fungal populations. *R. secalis* populations were collected from 31 individual fields and four regional sites in 14 countries from five continents (Table 1). The sampled regions included the Middle East and Ethiopia, which represent the center of origin, and a major center of genetic diversity of the host, respectively. The five Australian populations (AUS), the two Norwegian

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(NOR), the Finnish (FIN), as well as the Californian (CAL) populations were described previously (32,40). The two South African (SA) and six of the seven Swiss (CH) collections and the Ethiopian (ET) collections were described earlier (27). Swiss collection 7, here reported as CH1, was isolated from a field of rye. Collections from Eritrea (ERI), Syria (SYR), and Jordan (JOR) are reported here for the first time, as are the collections from France (FRA), Germany (GER), Turkey (TUR), and the United Kingdom (UK). The latter collections from Turkey and Europe were single isolate collections originating from many fields and were not considered to represent discrete field populations. Instead, these were considered to represent large geographical regions.

Isolation and DNA extraction. For the isolation and culturing of the Eritrean, Ethiopian, Swiss, Syrian, and Jordanian collections, as well as for the single isolate collections (FRA, GER, TUR, and UK), we followed the same methods described for previously studied populations as described in McDonald et al. (32). Spores from each isolate were transferred into 1.8-ml Cryo-Tubes (Nunc CryoLine System, Denmark) containing silica gel

(Fluka Chemie GmbH, Germany) for long-term storage at -80°C . An exception to the methods mentioned above was that DNA for these collections was extracted with the DNeasy Plant Mini DNA extraction kit (Qiagen GmbH, Germany) according to the specifications of the manufacturer.

Marker analysis. From each isolate, 3.5 to 5 μg of DNA was digested overnight at 37°C with 50 units of *EcoRI* (Amersham Pharmacia Biotech). The DNA fragments were electrophoresed on 0.8% agarose gels in $1\times$ Tris-borate-EDTA (TBE) buffer at 75 V for 14 h. Southern blotting, nick translation with ^{32}P -labeled dCTP (Amersham Bioscience UK Limited), and hybridization were as described previously (5). Anonymous DNA probes used for RFLP analysis were described earlier (32,40). The seven probes used were pRS2, pRS6, pRS26, pRS37, pRS47, pRS49, and pRS52. Probe pRS26 was previously used as a DNA fingerprint probe for Californian and Australian populations (32,40). When applied to the Eritrean, Ethiopian, Swiss, or Syrian *R. secalis* isolates, pRS26 did not show a high degree of polymorphism and only two to four fragments could be observed. An

TABLE 1. Description of *Rhynchosporium secalis* populations

Countries	Locations	Population	Sampling strategy	Cultivar	Total sampling area	Sampling area represented by leaves	Source and year of collection or previous publication
Africa							
Eritrea		ERI	Hierarchy	Unknown landrace	500 m ²	10 m ²	A. Yahyaoui, 2002
Ethiopia	Edagahamus	ET1	Hierarchy	Unknown local barley cultivars	400 m ²	8 m ²	K. Meles, 2001
	Atsbi	ET2	Hierarchy	Unknown local barley cultivars	400 m ²	8 m ²	K. Meles, 2001
	Adineba	ET3	Hierarchy	Unknown local barley cultivars	200 m ²	8 m ²	K. Meles, 2001
	Zata	ET4	Hierarchy	Unknown local barley cultivars	400 m ²	8 m ²	K. Meles, 2001
South Africa	Bredasdorp	SA1	Transects	Unknown barley cultivar	15 m ²	...	Linde et al. (27)
	Langewens	SA2	Transects	Different barley F2 crosses	30 m ²	...	Linde et al. (27)
Australia	Wagga Wagga	AUS1	Transects	Clipper	4.3 m ²	...	McDonald et al. (32)
	Methul	AUS2	Hierarchy	O'Conner	200 m ²	6 m ²	McDonald et al. (32)
	Horsham	AUS3	Hierarchy	Arapolis	200 m ²	6 m ²	McDonald et al. (32)
	Quindanning	AUS4	Hierarchy	Stirling	200 m ²	6 m ²	McDonald et al. (32)
	Rannock	AUS5	Hierarchy	Schooner	200 m ²	6 m ²	McDonald et al. (32)
Europe							
Finland	Jokioinen	FIN	Hierarchy	Kymppi	500 m ²	10 m ²	Salamati et al. (40)
France		FRA	Random	Unknown barley cultivars	Random collections	...	J. Speakman, 1997
Germany		GER	Random	Unknown barley cultivars	Random collections	...	J. Speakman, 1997
Norway	Buskerud	NOR1	Hierarchy	Tyra	500 m ²	10 m ²	Salamati et al. (40)
	Stjørdal	NOR2	Transects	Tyra	20 m ²	...	Salamati et al. (40)
Switzerland	Zofingen	CH1	Hierarchy	Organic rye cv. Danko	300 m ²	8 m ²	B. A. McDonald, 1999
	Leuggern	CH2	Hierarchy	Unknown barley cultivar	300 m ²	8 m ²	Linde et al. (27)
	Lucens	CH5	Hierarchy	Unknown cultivars of two-component barley mixture	300 m ²	8 m ²	Linde et al. (27)
	Cugy	CH6	Hierarchy	Unknown barley cultivar	300 m ²	8 m ²	Linde et al. (27)
	Zürich	00CH	Unordered transects	Experimental barley plots	800 m ²	36 m ²	Linde et al. (27)
	Eschikon	01CH1	Transects	Baretta	100 m ²	...	Linde et al. (27)
	Dietikon	01CH2	Transects	Unknown organic barley cultivar	120 m ²	...	Linde et al. (27)
UK		UK	Random	Unknown barley cultivars	Random collections	...	J. Speakman, 1997
Middle East							
Jordan	Al Raba	JOR1	Hierarchy	Unknown barley cultivar	400 m ²	8 m ²	K. Meles, 2001
	Al Raba	JOR2	Hierarchy	Unknown barley cultivar	400 m ²	8 m ²	K. Meles, 2001
Syria	Batajik	SYR1	Unordered transects	Rihane	750 m ²	...	C. C. Linde, B. A. McDonald, 2002
	Batajik	SYR2	Hierarchy	Unknown barley cultivar	400 m ²	8 m ²	C. C. Linde, B. A. McDonald, 2002
	Al Jurn	SYR3	Unordered transects	Furat	1,000 m ²	...	C. C. Linde, B. A. McDonald, 2002
	Al Aswad	SYR4	Unordered transects	Arabi Aswad	600 m ²	...	C. C. Linde, B. A. McDonald, 2002
	Beylounan	SYR5	Unordered transects	ICARDA landrace FTP00-267	1,050 m ²	...	C. C. Linde, B. A. McDonald, 2002
	Aim Issa	SYR6	Random	<i>Hordeum spontaneum</i>	40,000 m ²	...	C. C. Linde, B. A. McDonald, 2002
Turkey		TUR	Random	Unknown barley cultivars	Random collections	...	J. Speakman, 1997
United States	California	CAL	Subsample of a hierarchy	Barley composite cross	200 m ²	<50 m ²	McDermott et al. (30)

alternative DNA fingerprint had to be developed for these populations. Therefore, 45 Operon RAPD primers were screened for polymorphisms against five *R. secalis* isolates from Switzerland. The Operon primer OPL11 gave a unique banding pattern for all five isolates and produced 17 polymorphic RAPD amplicons that could be combined with single loci RFLP and mating type data to differentiate among genotypes in populations. Polymerase chain reactions (PCR) were carried out in 20- μ l volumes containing 2 μ l of 10 $\times$ PCR buffer, 0.1 mM of each dNTP, 0.3 μ M Operon primer OPL11, 1 unit of *Taq* polymerase (Amersham Pharmacia Biotech Inc.), and 5 μ l of genomic DNA (5 to 15 ng final DNA concentration). PCR conditions included a denaturing step at 96°C for 2 min, followed by 40 cycles at 94°C for 20 s, 34°C for 20 s, and 72°C for 2 min. Finally, a 12-min PCR extension was carried out at 72°C. Amplification products were electrophoresed on 1.5% (wt/vol) agarose gels in 0.5 $\times$ TBE buffer at 70 V for 15 h. The gels were stained in ethidium bromide (7 μ g/liter) for 15 min and destained in distilled water for 20 min. Bands were visualized on a 312 nm UV transilluminator.

Multiplex PCR of mating type locus. Mating types of the isolates in the previously uncharacterized populations were determined using multiplex PCR as described earlier (27).

Data analysis. The different restriction fragment size variants detected by each probe were treated as alleles at each RFLP locus. Only unambiguously scored alleles were included in the analysis. For the RAPD fingerprinting, the presence or absence of unambiguous amplicons was recorded. The data obtained for the RFLP loci, the RAPD fingerprint, and the mating types were combined to identify clones. Isolates having the same RFLP haplotype and the same RAPD pattern for the 17 polymorphic amplicons, as well as the same mating type, were assigned to the same multilocus haplotype and assumed to represent individual members of the same clone. RAPD data were used only to identify clones within fields. RAPD data were not used to compare populations. For each clone, only one representative was chosen to construct a clone-corrected data set. Clone correction was applied to each field collection.

We grouped populations as follows: Ethiopia (ET) and Eritrea (ERI) were pooled as the East African region (EA), Syria (SYR) and Jordan (JOR) as the Middle East (ME), the five Australian populations as (AUS), Norway (NOR) and Finland (FIN) as Scandinavia (SCA), the Swiss populations as (CH), and the CH and SCA populations were further combined as Europe (EU). During analysis we realized that the Swiss population CH1 from rye differed significantly from all other barley populations as described later. Therefore, we excluded CH1 from analyses in which populations were pooled. For population comparisons, only data from the shared RFLP loci (pRS6, pRS37, pRS47, pRS49, and pRS52) were included in the analyses. Field collections from Germany (GER), France (FRA), the United Kingdom (UK), and Turkey (TUR) were sampled from fields across a wide geographical area with one to two isolates collected from each field. Because these isolates did not represent discrete field populations, they were only added to the cluster analysis to represent regional populations, and these collections shared only four RFLP loci (pRS6, pRS37, pRS47, and pRS49) with all other characterized populations.

Genetic variation within populations. Estimates of genotypic diversity for each population were based on the Shannon-Wiener index H' (42). Only one isolate per leaf was included in this analysis, unless the two isolates from the same leaf were different genotypes. To measure evenness of distribution of genotypes within a population, H' was normalized for variable sample size by scaling the index by $H'_{\max}$, which is the maximum genotypic diversity for a given sample size expressed here as the logarithm of the sample size $\ln(n)$ (15,43). Scaling H' by the sample size could bias the estimation of evenness. Therefore, H' was also scaled by the number of observed genotypes (g_{obs}) (18). For all other analyses, data sets were clone-corrected to prevent over-

representation of alleles in frequently occurring clones. All analyses were conducted with PopGene (version 1.32; F. C. Yeh, R. C. Yang, and T. J. B. Boyle, Molecular Biology and Biotechnology Centre, University of Alberta, Canada) unless otherwise specified.

Nei's gene diversity (34) was measured for all individual populations, as well as for populations that were combined to represent larger geographic areas. The total gene diversity was partitioned into different spatial components to determine how much genetic variation could be attributed to each spatial scale in the hierarchy (4,34). At the macrogeographical level, we calculated the amount of genetic diversity distributed among regions, among fields within regions, and within fields within regions. At the microgeographical level, we included only the 17 hierarchically sampled field populations and the diversity was partitioned among fields, among sampling sites within fields, and within sampling sites within fields.

We compared the allele richness of regional populations by calculating the number of alleles per locus. Because regional populations consisted of different sample sizes, we standardized population sizes by resampling (25,54) based on the size of the smallest regional population ($CAL = 26$ and $SA = 40$). Allele numbers and Nei's gene diversity were calculated by nonparametric bootstrap analysis as the mean value of 100 resamples with replacement using the Resampling Stats software package (version 5.02, 2000; Resampling Stats, Arlington, VA) and evaluated by analysis of variance implemented in Systat (version 10 for Windows; Systat Software Inc., Richmond, CA). The post-hoc least squares means (LSM, Tukey) test was performed in Systat (version 10) to compare allele richness and gene diversity values of regional populations.

Random mating. Analysis of multilocus associations (6) was used to measure the extent of gametic disequilibrium. We also calculated the index of association, I_A (6) based on 1,000 artificially recombined data sets using MultiLocus, version 1.2 (1). Significant departures from an expected 1:1 ratio in mating type frequencies were tested with a χ^2 test (11) for populations that were not characterized previously in Linde et al. (27).

Genetic variation among populations. Differences in allele frequencies among populations were calculated with a contingency χ^2 test (53). To reduce type 1 error, the Bonferroni correction (50) was applied to all χ^2 tests. Unbiased measures of genetic distance were calculated among all populations according to Nei (35).

The degree of population subdivision (G_{ST}) and gene flow (Nm) were calculated within and among each geographic region (47). Isolation by distance was determined for each pair of populations (46). Population subdivision was also measured with Weir and Cockerham's Θ (51). The Θ values from 1,000 artificial data sets, which were generated assuming no population subdivision, were compared with the observed Θ using MultiLocus.

Pairwise genetic distance among populations was measured using Nei's genetic distance, D_A (35), using the DISPAN program (T. Ota, Pennsylvania State University). A cluster analysis was performed using an unweighted pair group method using arithmetic mean (UPGMA) phenogram based on Nei's genetic distances, D_A (35), as implemented in DISPAN including 1,000 bootstrapped replicates.

RESULTS

Genotypic diversity within regions. Among the 1,366 *R. secalis* isolates analyzed, 955 distinct genotypes were found (Table 2). Thus, 70% of all isolates were unique haplotypes. Genotypic diversities and evenness in most field populations from Australia, Eritrea, Ethiopia, Switzerland, and Norway were high ($H'/H'_{\max} > 0.80$, $H'/g_{\text{obs}} > 0.83$), independent of the number of RFLP loci analyzed (Table 2). The lowest genotypic diversity values were observed in Syria. Genotypic diversity was not calculated for the

Syrian population SYR5, due to its small sample size. No haplotypes were shared among different populations.

Gene diversity within regions. Across the five RFLP loci shared among all field populations, the number of alleles ranged from 1.4 to 4.2 with an average of 2.6 (Table 2). Private alleles (pa) were found in Germany (1 pa), Syria (1 pa), Eritrea (2 pa), Finland (2 pa), Switzerland (3 pa, all from CH1), and Norway (8 pa). Gene diversity within fields ranged from 0.17 to 0.53 (Table 2), with the populations from Eritrea having the lowest average gene diversity (0.22) and the populations from Norway having the highest (0.51) average gene diversity. We performed bootstrap analyses with standardized population sizes of both 26

and 40, representing the smallest regional population sizes in California and South Africa, respectively. This analysis showed that Scandinavia had significantly ($P \leq 0.05$) higher allele richness than the other regions analyzed (Table 3) while South Africa had the fewest alleles per locus. This pattern was the same for both population size variants (Table 3). Switzerland had the second-highest allele richness (Table 3).

The majority of the total global genetic diversity was distributed within a field. In the macrogeographical comparison that included 30 field populations, 58% of the total genetic diversity was located within fields, 11% existed among fields within regions, and 31% was distributed among regions (Table 4). At the

TABLE 2. Summary of genetic diversity parameters for *Rhynchosporium secalis* field collections from 14 countries on five continents

Countries	Population	No. of strains	No. of observed haplotypes g_{obs}	No. of RFLP loci	No. of private alleles	H^v	H'/H_{max}^w	H'/g_{obs}^x	All available loci		No. of shared RFLP loci ^z	Shared loci ^z	
									Average no. of RFLP alleles/locus	Gene diversity ^y		Average no. of RFLP alleles/locus	Gene diversity ^y
Africa													
Eritrea	ERI	27	25	6	2	3.19	0.97	0.99	1.7	0.18	5	1.8	0.22
Ethiopia	ET1	30	28	5	...	3.31	0.97	0.99	2.8	0.33	5	2.8	0.33
	ET2	14	12	5	...	2.40	0.91	0.97	2.2	0.31	5	2.2	0.31
	ET3	32	30	5	...	3.20	0.92	0.97	2.4	0.22	5	2.4	0.22
	ET4	33	27	5	...	3.38	0.98	0.99	2.2	0.26	5	2.2	0.26
	...	...	...	...	...	...	...	...	2.4	0.28	...	2.4	0.28
South Africa	SA1	21	12	6	...	2.24	0.74	0.90	1.8	0.27	5	1.8	0.25
	SA2	33	28	6	...	3.27	0.94	0.98	2.5	0.43	5	2.6	0.41
	...	...	...	...	...	...	...	...	2.2	0.35	...	2.2	0.33
Australia													
	AUS1	66	49	8	...	3.56	0.85	0.91	2.6	0.55	5	2.4	0.41
	AUS2	57	51	8	...	3.89	0.96	0.99	3.1	0.52	5	2.8	0.42
	AUS3	39	29	8	...	3.23	0.88	0.96	2.5	0.51	5	2.4	0.42
	AUS4	42	25	8	...	3.07	0.82	0.95	2.7	0.54	5	2.8	0.49
	AUS5	61	60	8	...	4.09	0.99	1.00	2.6	0.51	5	2.6	0.41
	...	...	...	...	...	...	...	...	2.7	0.53	...	2.6	0.43
Europe													
Finland	FIN	82	63	9	2	4.05	0.92	0.98	4.4	0.46	5	4.2	0.48
France	FRA	3	3	5	...	...	...	...	...	...	...	...	...
Germany	GER	18	18	5	1	...	...	...	...	...	...	...	...
Norway	NOR1	46	43	9	5	3.73	0.97	0.99	5.3	0.46	5	4.2	0.53
	NOR2	140	45	9	3	3.61	0.73	0.94	4.3	0.45	5	3.4	0.49
	...	...	...	...	...	...	...	...	4.8	0.46	...	3.8	0.51
Switzerland	CH1	23	21	6	3	3.01	0.96	0.99	1.8	0.19	5	2.0	0.22
	CH2	35	25	6	...	3.05	0.86	0.95	2.8	0.32	5	3.0	0.37
	CH5	27	20	6	...	2.92	0.89	0.97	2.0	0.27	5	2.4	0.32
	CH6	32	30	6	...	3.38	0.98	0.99	2.8	0.32	5	3.0	0.35
	00CH	48	44	6	...	3.74	0.97	0.99	3.8	0.42	5	4.2	0.50
	01CH1	33	30	6	...	3.37	0.96	0.99	2.5	0.31	5	2.6	0.29
	01CH2	43	37	6	...	3.54	0.94	0.98	2.3	0.31	5	2.4	0.28
	...	...	...	...	...	...	...	2.6	0.31	...	2.8	0.33	
UK	UK	10	10	5	...	...	...	...	...	...	...	...	...
Middle East													
Jordan	JOR1	17	9	6	...	5.25	0.68	0.88	1.3	0.18	5	1.4	0.19
	JOR2	28	16	6	...	2.60	0.78	0.94	1.8	0.19	5	2.2	0.27
	...	...	...	...	...	...	...	...	1.6	0.19	...	1.8	0.23
Syria	SYR1	52	16	6	...	2.35	0.60	0.85	1.5	0.20	5	1.6	0.23
	SYR2	53	22	6	1	2.56	0.64	0.83	2.8	0.30	5	3.0	0.35
	SYR3	70	37	6	...	3.28	0.77	0.91	2.0	0.22	5	2.2	0.27
	SYR4	55	30	6	...	3.14	0.78	0.92	1.7	0.14	5	1.8	0.17
	SYR5	6	6	6	...	...	...	...	1.5	0.24	5	1.6	0.29
	SYR6	36	26	6	...	3.02	0.84	0.93	2.0	0.26	5	2.2	0.31
	...	...	...	...	...	...	...	1.9	0.23	...	2.0	0.27	
Turkey	TUR	2	2	5	...	...	...	...	...	...	...	...	...
United States	CAL	52	26	7	...	3.01	0.76	0.90	3.1	0.55	5	3.4	0.41
Total		1,366	955	...	17	...	...	...	2.6	0.34	...	2.6	0.35

^v Shannon-Wiener diversity index (42).

^w Normalized Shannon-Wiener index. H' is scaled by $H_{max} = \ln(n)$, where n is the number of collected strains of the sample.

^x Normalized Shannon-Wiener index. H' is scaled by the observed numbers of haplotypes (18).

^y Nei's gene diversity (34).

^z The parameters were calculated based on five restriction fragment length polymorphism (RFLP) loci (pRS6, pRS37, pRS47, pRS49, and pRS52) that were shared among the populations.

microgeographical level that included 17 field populations, 39% of the global genetic diversity was distributed within a plot of 1 m² within a field, 16% was distributed among plots within a field, 13% among fields within a region, and 32% among regions (Table 4).

Population subdivision and gene flow within regions. We tested the magnitude of subdivision among populations from previously undescribed regions by testing for homogeneity in gene frequencies using a contingency χ^2 test (53). Populations from Scandinavia and Australia were analyzed previously (32,40). The null hypothesis of equal allele frequencies was rejected for all new regional populations. These results were unchanged following Bonferroni correction. We applied the test for pairwise comparisons between populations from the same country to determine which populations led to the rejection of the null hypothesis. No significant differences were found for any loci between CH5 and CH6, ET2 and ET3, ET3 and ET4, SYR1 and SYR2, SYR1 and SYR3, and JOR1 and JOR2. The remaining pairwise comparisons differed at some loci but never across all loci.

Population subdivision between the Swiss population collected from rye (CH1) and all other Swiss populations was high (Table 5) with an average G_{ST} of 0.59. Corresponding Nm among population CH1 and other populations was always lower than 0.5 (Table 5). The high genetic distance between CH1 and other populations is shown in the genetic distance phenogram (Fig. 1). Low population subdivision existed between the remaining Swiss populations, with the exception of 01CH2, which deviated from the other Swiss barley populations with a higher G_{ST} ranging from 0.24 to 0.31 (Table 5). Low population subdivision ($G_{ST} = 0.03$ to 0.09) was observed among Ethiopian populations (Table 5). The overall G_{ST} in Ethiopia and South Africa was low (Table 6). In the Middle East, the G_{ST} ranged from 0.02 to 0.20 in Syria, and was 0.12 between the two populations from Jordan (Table 5).

Gametic equilibrium and mating system. Brown's multilocus analysis showed that 13 out of 31 populations departed from gametic equilibrium (Table 7). Ten populations departed from gametic equilibrium using the index of association (I_A).

To complement analysis of mating type frequencies published in a previous study (27), populations from Syria, Jordan, and Eritrea were analyzed for mating type frequencies and tested for deviations from a 1:1 ratio with a χ^2 analysis. None of the clone-corrected populations deviated significantly from a 1:1 ratio (Table 7).

Population subdivision and gene flow between regions. The G_{ST} and the Θ across all populations was 0.42 and 0.37, respectively (Table 6). Among regions, the average Nm ranged from less than one to almost four migrants per generation (Table 5). The genetic distance phenogram (Fig. 1) placed populations from Switzerland in one clade, except population CH1 from rye and population 01CH2. The latter clustered with the South African populations, which were genetically closer to the Australian populations and the isolates collected across Germany, the United Kingdom, France, and Scandinavia. The East African populations clustered separately but closest to populations from the Middle East and California.

Isolation by distance. The correlation coefficient between the logarithm of Nm and the geographical distance among 31 populations was -0.39 ($P < 0.001$). When Swiss population CH1 was excluded from this analysis the correlation coefficient was -0.49 , which was significant at $P < 0.001$ (Fig. 2).

DISCUSSION

Genetic structure, population subdivision, and gene flow. Previous studies based on RFLP markers showed that a high level of genetic diversity was distributed on a small spatial scale

TABLE 3. Allele richness and average gene diversity (34) after standardizing *Rhynchosporium secalis* populations from six different regions to a population size of 26 or 40^w

Region	$N = 26$				$N = 40$			
	Allele richness ^x	SD ^y	H^z	SD ^y	Allele richness ^x	SD ^y	H^z	SD ^y
Australia	2.80 d	0.17	0.45 c	0.03	2.93 c	0.17	0.45 b	0.03
East Africa	2.56 e	0.22	0.32 d	0.03	2.71 d	0.20	0.32 c	0.03
California	2.95 c	0.27	0.51 ab	0.04	...	...	...	...
Middle East	2.61 e	0.28	0.35 d	0.05	2.94 c	0.28	0.36 c	0.04
Scandinavia	4.20 a	0.41	0.53 a	0.03	4.60 a	0.36	0.53 a	0.03
South Africa	2.38 f	0.16	0.47 bc	0.04	2.49 e	0.13	0.47 b	0.03
Switzerland	3.51 b	0.33	0.53 a	0.04	3.76 b	0.37	0.53 a	0.03

^w N represents standardized population size for resampling. Column values followed by the same letter are not significantly ($P \leq 0.05$) different based on a post-hoc least squares means test for multiple comparisons. The resampling procedure was repeated 100 times.

^x Average alleles per locus.

^y Standard deviation based on resampling (Resampling Stats, Arlington, VA; version 5.02, 2000).

^z Nei's gene diversity (34).

TABLE 4. Hierarchical distribution of gene diversity among *Rhynchosporium secalis* populations from five continents

	Macrogeographical level				Microgeographical level				
	Percentage of total H^x				Percentage of total H^x				
	Number of populations	Within fields	Among fields within regions	Among regions	Number of populations	Within plots within fields	Among plots within fields	Among fields within regions	Among regions
Australia	5	94%	6%	...	4	76%	18%	6%	...
East Africa	5	87%	13%	...	4	61%	30%	9%	...
Middle East	8	84%	16%	...	3	47%	34%	19%	...
Scandinavia	3	96%	4%	...	2	70%	28%	2%	...
South Africa	2	94%	6%	...	...	...	...	...	...
Switzerland	6	75%	25%	...	4	45%	40%	15%	...
Global	30 ^y	58%	11%	31%	17 ^z	39%	16%	13%	32%

^x Nei's gene diversity (34).

^y Includes Californian *R. secalis* population but excludes Swiss rye population CH1.

^z Includes hierarchically sampled *R. secalis* populations only.

for the Scandinavian and Australian *R. secalis* populations (32, 40). Low population subdivision consistent with high gene flow was observed within those regions (32,40). We obtained similar results from four additional barley-growing regions in this study.

The great majority (58% globally) of genetic variation for all surveyed regions was distributed within fields. The highest amount (25%) of genetic differentiation among regional barley *R. secalis* field populations was found in Switzerland. This may reflect the fact that these fields were sampled over a 4-year period. Nearly 40% of the global gene diversity was distributed within a 1-m² area in fields around the world. Though the total area in each field represented by the hierarchically collected leaves was small (≈8 m²), a large number of different genotypes were observed across this sampling area on all continents. Despite distances between fields within regions ranging up to thousands of kilometers, populations within regions were mostly similar, though pairwise comparisons of allele frequencies between regional populations showed that there were some differences. On the other hand, populations from different regions mostly had larger genetic distances, with population differentiation increasing with geographical distance.

Considering all of these findings, we conclude that there is significant gene flow on a regional scale, and more restricted gene flow between regions. This suggests that there is regular movement of inoculum over a spatial scale of at least 100 km. This inoculum could be carried on infected seed (19,21,22,26,45). We hypothesize that it is equally plausible that the inoculum responsible for the observed high degree of similarity on a regional scale

is wind-dispersed ascospores from the unknown sexual stage of *R. secalis*. Using a stepping-stone model of dispersal, the putative ascospores would need to move only a few kilometers at a time to link together barley fields over large areas within barley-growing regions.

Some populations and isolate collections (e.g., 01CH2, GER, UK, FRA, and CAL) did not fit the overall pattern of isolation by distance. We consider it likely that man-mediated gene flow played a significant role in these exceptions. For instance, the Australian populations cluster together with those from the United Kingdom and Germany, and are closely related to the South African populations. Exchange of infected breeding material and/or seeds between countries of the British Commonwealth could have played a significant role in creating these relationships, especially during the British colonial period when barley and probably *R. secalis* on infected barley seed were introduced into new areas.

Recombination. Until the teleomorph of *R. secalis* is identified, recombination can only be inferred by indirect methods such as measures of genotype diversity, mating type frequencies, and associations among alleles, as reviewed by Milgroom (33) and Taylor et al. (48). There are several lines of evidence that indicate regular sexual reproduction in *R. secalis* populations around the world. The majority of the populations in this and other studies (32,40) had intermediate to high genotypic diversities which is not expected for an exclusively asexual (mitosporic) fungus. Populations from Australia, Scandinavia, and California were analyzed previously (32,40) for multilocus association (6), and the majority of these populations were in gametic equilibrium.

TABLE 5. Population differentiation (G_{ST}) (below diagonal), and gene flow (Nm) (above diagonal) among *Rhynchosporium secalis* populations from Australia (AUS), California (CAL), East Africa (EA, ERI, and ET1-ET4), the Middle East (ME, JOR1, JOR2, and SYR1-SYR6), Scandinavia (SCA, FIN, NOR1, and NOR2), South Africa (SA), and Switzerland (CH and CH1-01CH2)^z

Population	SYR1	SYR2	SYR3	SYR4	SYR5	SYR6	JOR1	JOR2
SYR1	...	13.71	22.43	3.45	5.35	7.20	3.03	3.68
SYR2	0.04	...	7.57	3.09	7.69	7.24	2.34	3.34
SYR3	0.02	0.06	...	7.16	6.28	13.08	5.22	7.72
SYR4	0.13	0.14	0.07	...	3.58	4.78	7.13	5.65
SYR5	0.09	0.06	0.07	0.12	...	6.37	1.95	3.18
SYR6	0.06	0.06	0.04	0.09	0.07	...	2.73	7.15
JOR1	0.14	0.18	0.09	0.07	0.20	0.15	...	3.65
JOR2	0.12	0.13	0.06	0.08	0.14	0.07	0.12	...

Population	CH1	CH2	CH5	CH6	00CH	01CH1	01CH2
CH1	...	0.35	0.29	0.34	0.44	0.33	0.34
CH2	0.59	...	7.95	7.18	4.80	6.53	1.36
CH5	0.63	0.06	...	18.26	2.32	2.79	1.12
CH6	0.60	0.07	0.03	...	2.91	3.54	1.33
00CH	0.53	0.09	0.18	0.15	...	3.05	1.32
01CH1	0.60	0.07	0.15	0.12	0.14	...	1.62
01CH2	0.60	0.27	0.31	0.27	0.28	0.24	...

Population	CH	EA	ME	SA	SCA	CAL	AUS
CH	...	0.63	0.64	0.94	1.12	1.03	0.85
EA	0.44	...	0.78	0.71	1.27	1.46	0.89
ME	0.44	0.39	...	0.98	1.67	2.57	0.88
SA	0.35	0.41	0.34	...	1.75	1.59	2.49
SCA	0.31	0.28	0.23	0.22	...	3.71	2.35
CAL	0.33	0.26	0.16	0.24	0.12	...	2.62
AUS	0.37	0.36	0.36	0.17	0.18	0.16	...

Population	ET1	ET2	ET3	ET4	ERI
ET1	...	4.82	4.88	8.72	3.85
ET2	0.09	...	14.30	7.63	0.10
ET3	0.09	0.03	...	14.29	2.70
ET4	0.05	0.06	0.03	...	2.98
ERI	0.12	0.10	0.16	0.14	...

^z AUS, CAL, CH, EA, ME, SCA, and SA represent regions for which the populations were pooled.

Gametic equilibrium was also found in the majority of the 22 additional populations analyzed in this study. More evidence for the occurrence of sexual reproduction comes from the observed 1:1 ratio in mating type frequencies, consistent with frequency-dependent selection on mating types which is a good indication of random mating (29). Three populations (AUS4, CAL, and 01CH2)

that deviated significantly from equal mating type frequencies (27) were also in gametic disequilibrium.

The primary geographical exception to the global pattern of gametic equilibrium was Switzerland, where four of seven field populations showed significant disequilibrium for both tests, but only one of these populations showed a departure from the 1:1 ratio in mating types. We cannot explain these findings. But by interviewing one of the farmers, we learned that his field was planted to a mixture of new, fungicide-treated seed and old, untreated seed saved from a field that had been heavily infected by *R. secalis* in the previous year. In this case, it is possible that the *R. secalis* population in our sample was a mixture of two different populations, one originating from infected seed of the previous year and the second originating from leaf infections by putative ascospores in the sampled year. If these two source populations differed in their RFLP allele frequencies, this could have generated the observed disequilibrium.

R. secalis has been shown to be closely related to species of *Tapesia* and its anamorph *Ramulispora* in a phylogenetic study (13). Two species of *Tapesia*, *T. yallundae* and *T. acuformis*, cause eyespot on wheat. One might thus expect that *R. secalis* would have a population genetic structure similar to these *Tapesia* spp. that also infect cereals. Sexual fruiting bodies of *T. yallundae* and *T. acuformis* have been observed in several wheat producing regions (28), although it is not clear how much the sexual stage contributes to the disease cycle of these two pathogens (9,10). However, sexual reproduction seems to maintain high levels of genetic diversity and to prevent divergence of regional populations, possibly due to long-distance dispersal of ascospores (9,10). Furthermore, tests for mating type ratios and associations of alleles at unlinked loci suggest gametic equilibrium as expected for randomly mating populations (9,10). Similar population structures were found for *R. secalis*, i.e., high levels of genetic diversity, low population subdivision, equal mating type ratios, and gametic equilibrium. But no teleomorph has yet been observed for *R. secalis*.

Center of origin. The center of origin of barley is in the Fertile Crescent (20,55) and Ethiopia is a center of diversity thought to represent an early place for domestication and a secondary center of origin for barley (20). Other cultivated cereals, including wheat, rye, and oats, also originated in the Fertile Crescent (20,55). In another well-studied example of a leaf spotting cereal pathogen, *Mycosphaerella graminicola*, RFLP and sequence data were consistent with the hypothesis that the host and pathogen did indeed coevolve at the host's center of origin (2,54). In these studies, populations from Israel showed the highest levels of gene

Fig. 1. Unweighted pair-group method with arithmetic average phenogram based on Nei's genetic distance D_A (35) of *Rhynchosporium secalis* populations from five continents based on four shared restriction fragment length polymorphism loci. Bootstrap values are based on 1,000 replicates. Only bootstrap values >50% are shown.

TABLE 6. Measures of population subdivision (G_{ST} , Θ) and gene flow (Nm) in *Rhynchosporium secalis* populations from five continents

Regions	Number of populations	G_{ST}	Nm	Θ	P^y	Geographic distance
Australia	5	0.07	6.68	0.05	<0.001	50–2,500 km
Australia without AUS4	4	0.05	10.27	0.03	<0.001	50–500 km
East Africa	5	0.13	3.21	0.13	<0.001	20–300 km
Ethiopia	4	0.09	4.89	0.08	<0.001	20–200 km
Europe	9	0.30	1.16	0.29	<0.001	10–2,000 km
Scandinavia	3	0.08	5.96	0.09	<0.001	600–900 km
Switzerland without CH1	6	0.26	1.46	0.27	<0.001	10–200 km
Switzerland without CH1 and 01CH2	5	0.17	2.53	0.17	<0.001	10–200 km
Norway	2	0.09	5.26	0.14	<0.001	600 km
Middle East	8	0.16	2.70	0.10	<0.001	0.5–500 km
Jordan	2	0.12	3.65	0.12	1.00	2 km
Syria	6	0.12	3.75	0.09	<0.001	0.1–150 km
South Africa	2	0.06	7.29	0.04	0.127	200 km
Global ^z	30	0.42	0.69	0.37	<0.001	0.1–17,000 km

^y P values of Θ (1,51). Significance values were obtained from 1,000 randomized data sets assuming no population subdivision.

^z In the global measurements, all populations were included except CH1.

diversity and allele richness. If *R. secalis* coevolved with wild barley prior to domestication or very early in the domestication process, we would expect to find the highest genetic diversity in the Syrian and Jordanian populations followed by the Ethiopian populations. We tested this hypothesis by comparing the allele richness and genetic diversity across regions. Bootstrap analysis showed that the highest allele richness was found in Scandinavia (4.60) followed by Switzerland (3.76). Significantly lower values were found in the putative centers of origin of barley, as well as in all other regions. The same pattern was observed for gene diversity, except that California was not significantly different from Scandinavia and Switzerland. Furthermore, most of the private

alleles were found in the Scandinavian population (10 pa), with only one private allele in Syria and two in Eritrea. The data available to us suggest that *R. secalis* did not originate in the Middle East or Ethiopia. Our findings are more consistent with the alternative hypothesis that *R. secalis* originated somewhere in northern Europe.

Other cases have been proposed where the pathogen and host did not coevolve in the same center of origin (12,44). If *R. secalis* originated in northern Europe, then cultivated barley was not its original host and it likely coevolved with a different host, perhaps a wild *Hordeum* relative or other endemic grass, and then made a host jump after barley was introduced into Europe. We were

TABLE 7. Tests for multilocus associations and mating type ratios for 31 *Rhynchosporium secalis* populations from five continents

	Populations	Multilocus association						Mating type frequencies				
		m^r	n^s	$S^2_k{}^t$	L^u	I_A^v	P^v	MAT1-1 ^w	MAT1-2 ^w	χ^2 value ^x	χ^2 value ^y	Contingency χ^2z
Australia	AUS1	6	48	1.46	1.66	0.094	0.088	26 (34)	23 (41)	0.709	0.184	9.325 (4)
	AUS2	6	49	1.42	1.58	0.139	0.058	25 (25)	30 (30)	0.074	0.455	
	AUS3	6	26	1.57	1.81	0.044	0.401	16 (22)	20 (24)	0.000	0.444	
	AUS4	6	24	3.55*	2.18	1.225	<0.001	17 (35)	4 (13)	0.413	8.048**	
	AUS5	6	58	1.36	1.67	0.006	0.782	15 (16)	18 (26)	0.413	0.273	
Australia								99	95	...	0.082	
Eritrea	ERI	6	25	0.76	0.92	0.177	0.090	14 (15)	11 (12)	0.001	0.360	
Ethiopia	ET1	5	27	1.20*	1.18	0.260	0.031	11 (12)	16 (17)	0.002	0.926	4.353 (3)
	ET2	5	12	1.16*	1.12	0.299	0.070	5 (5)	6 (7)	0.033	0.091	
	ET3	5	28	0.59	0.77	0.051	0.314	13 (15)	17 (19)	0.004	0.533	
	ET4	5	27	0.76	0.85	0.192	0.309	16 (18)	8 (8)	0.403	2.667	
	Ethiopia							45	47	...	0.043	
Finland	Fin	6	62	1.29	1.64	-0.032	0.641	33 (50)	18 (21)	0.446	4.412*	
Jordan	JOR1	6	9	0.87*	0.82	0.224	0.155	6 (8)	3 (9)	0.910	1.000	1.963 (1)
	JOR2	6	16	1.44*	1.42	0.412	0.055	6 (11)	10 (17)	0.014	1.000	
	Jordan							12	13	...	0.040	
Norway	NOR1	6	43	1.19	1.58	-0.020	0.582	12 (13)	20 (21)	0.004	2.000	1.212 (1)
	NOR2	6	48	1.54	1.71	0.129	0.078	24 (65)	24 (60)	0.056	0.000	
	Norway							36	44	...	0.800	
South Africa	SA1	6	12	0.86	1.33	-0.220	0.976	31	28	...	0.153	0.396 (1)
	SA2	6	21	1.71	1.91	0.281	0.011	27	19	...	1.391	
	South Africa							58	47	...	1.152	
Switzerland	CH1	6	20	0.84	1.03	-0.190	0.957	10 (11)	11 (12)	0.002	0.048	15.097* (6)
	CH2	6	25	2.18*	1.42	1.312	<0.001	12 (20)	12 (12)	0.004	0.000	
	CH5	6	16	1.42*	1.37	0.500	0.161	8 (8)	17 (22)	0.189	3.240	
	CH6	6	29	1.85*	1.59	0.468	0.003	14 (14)	15 (15)	0.000	0.034	
	00CH	6	42	1.47*	1.42	0.582	<0.001	22 (25)	15 (15)	0.120	1.324	
	01CH1	6	29	1.33	1.51	0.329	0.010	14 (14)	11 (11)	0.000	0.360	
	01CH2	6	27	2.09*	1.49	0.676	<0.001	25 (31)	6 (6)	0.209	11.645**	
	Switzerland							105	87	...	1.688	
Syria	SYR1	6	16	0.94	1.10	-0.101	0.875	11 (33)	7 (19)	0.032	0.889	3.409 (5)
	SYR2	6	22	1.63*	1.56	0.514	0.017	13 (35)	12 (19)	1.177	0.040	
	SYR3	6	37	1.09	1.20	-0.039	0.579	22 (49)	16 (20)	1.889	0.947	
	SYR4	6	21	0.84	1.04	0.041	0.379	19 (37)	11 (20)	0.021	2.133	
	SYR5	6	5	1.61*	1.51	0.889	0.100	6	1	...	3.571	
	SYR6	6	26	1.01	1.29	0.080	0.252	14 (20)	13 (17)	0.030	0.037	
	Syria							85	60	...	4.310	
United States	CAL	5	26	2.17*	1.85	0.430	<0.010	6 (6)	18 (40)	2.812	6.000*	
Global								493	440			51.360** (30)

^r Number of loci.

^s Population size used in the analysis of multilocus association (6) and index of association, I_A (1.6).

^t Observed variance of the number of heterozygous comparisons. If S^2_k exceeds L , the population is not in gametic equilibrium as indicated by *.

^u Upper confidence limit.

^v The index of association (I_A) (1.6) was calculated for the observed populations and compared to 1,000 randomized data sets assuming panmixia.

^w Values in parentheses indicate number of mating type isolates MAT1-1 and MAT1-2 as determined by multiplex polymerase chain reaction (27) in the data set before it was clone-corrected.

^x χ^2 value based on a contingency χ^2 between clone-corrected and non-clone-corrected mating type ratios with 1 degree of freedom; all χ^2 values except the ones from Syria, Jordan, Eritrea, and CH1 are from Linde et al. (27).

^y χ^2 values based on a 1:1 ratio of mating type with 1 degree of freedom for the clone-corrected data set; * indicates mating type frequencies are significantly different at $P < 0.05$; all χ^2 values except the ones from Syria, Jordan, Eritrea, and CH1 are from Linde et al. (27).

^z χ^2 values based on contingency χ^2 analyses among populations. Numbers in parentheses indicate degrees of freedom; * indicates mating type frequencies among populations are significantly different at $P < 0.05$; ** indicates significantly different at $P < 0.01$; all χ^2 values except the ones from Syria, Jordan, Eritrea, and CH1 are from Linde et al. (27).

surprised to find that the allele richness and gene diversity in the Middle East, where barley has been cultivated for thousands of years, was not significantly higher than in Australia, South Africa, or California, where barley was introduced as a crop only in the last few hundred years. Additional populations from northern Europe and regions of the Middle East, as well as from South America and East Asia, should be included in future analyses to test the hypothesis that the center of origin was in northern Europe. In ways similar to the studies that were conducted for the potato late blight pathogen *Phytophthora infestans* in the central highlands of Mexico (17), other independent lines of evidence, such as pathogenic variability and host range, will be needed to unambiguously identify the center of origin of *R. secalis*.

Speciation in progress? The Swiss population CH1, originating from a rye field, was significantly different from all other Swiss populations that were sampled from barley. Several lines of evidence indicate this difference. A high genetic distance (Fig. 1) was found between CH1 and all *R. secalis* populations originating from barley. There was a high population differentiation (average $G_{ST} = 0.59$; $\Theta = 0.430$; $P < 0.001$), and a low number of shared alleles. Only 7 of the 34 alleles present at six RFLP loci across the Swiss barley-infecting populations were shared with the rye-infecting population of *R. secalis*. Furthermore, the three private alleles found in Switzerland were all from the rye-infecting population.

At least one migrant per generation exchanged between two populations is sufficient to prevent them from diverging by genetic drift. Less than one migrant per generation on average ($Nm = 0.35$) was exchanged between the rye-infecting and barley-infecting populations of *R. secalis* in Switzerland, which indicates that current gene flow neither limits genetic divergence of the fungus on different hosts, nor prevents random fixation of alleles due to genetic drift. Thus, the rye-infecting population of *R. secalis* appears to be genetically isolated from the barley-infecting population even though they coexist in the same region. We hypothesize from these lines of evidence that *R. secalis* on rye is diverging as a separate species, perhaps due to host specialization. Further investigations will be necessary to test the hypothesis that speciation is underway or has already occurred.

Applications. Breeders should consider the evolutionary risk posed by *R. secalis* when developing a strategy for choosing and deploying resistance genes (31). Our findings suggest that high levels of gene flow and recombination occur on a regional scale. Therefore, there is substantial risk for the regional breakdown of resistance genes (3) or for rapid spread of fungicide resistance (23). The high population subdivision existing among continents justifies the continuation of strict quarantine measures to exclude new genetic diversity from existing pathogen populations, espe-

cially those that have lower genetic diversity and thus a lower evolutionary potential. A center of origin for *R. secalis* in Scandinavia has consequences for barley breeding as additional resistance to *R. secalis* could be sought from native grasses in that area. The possibility of host specialization or perhaps ongoing speciation between *R. secalis* populations on barley and rye also has implications for breeding strategies in countries where rye is still an important crop. If host specialization exists, it will be necessary to determine whether genetic exchange can occur between the specialized populations. If so, rye-infecting *Rhynchosporium* populations could act as a reservoir of new virulence alleles for *R. secalis* on barley as avirulence genes might still be shared among the species, as is the case for *NIP1* (24,41).

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Fig. 2. Correlation between the logarithm of the geographical distance (m) and average number of migrants per generation (Nm) between each pair of *Rhynchosporium secalis* populations from five continents: $y = -0.245x + 1.796$, $R^2 = 0.249$.

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