Genetic Structure of *Phaeosphaeria nodorum* Populations in the North-Central and Midwestern United States

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Accepted for publication 13 August 2007.

ABSTRACT

Adhikari, T. B., Ali, S., Burlakoti, R. R., Singh, P. K., Mergoum, M., and Goodwin, S. B. 2008. Genetic structure of *Phaeosphaeria nodorum* populations in the north-central and midwestern United States. Phytopathology 98:101-107.

Stagonospora nodorum blotch, caused by *Phaeosphaeria nodorum*, is considered one of the most destructive foliar diseases of wheat in the United States. However, relatively little is known about the population biology of this fungus in the major wheat-growing regions of the central United States. To rectify this situation, 308 single-spore isolates of *P. nodorum* were analyzed from 12 populations, five from hard red spring wheat cultivars in Minnesota and North Dakota and seven from soft red winter wheat in Indiana and Ohio. The genetic structure of the sampled populations was determined by analyzing polymorphisms at five microsatellite or simple-sequence repeat (SSR) loci and the mating type locus. Although a few clones were identified, most *P. nodorum* populations had high levels of gene (H_S = 0.175 to 0.519) and genotype (D = 0.600 to 0.972) diversity. Gene diversity was higher among isolates collected from spring wheat cultivars in North Dakota and Minnesota (mean H_S = 0.503) than in those from winter wheat cultivars in Indiana and Ohio (H_S = 0.269). Analyses of clone-corrected data sets showed equal frequencies of both mating types in both regional and local populations, indicating that sexual recombination may occur regularly. However, significant gametic disequilibrium occurred in three of the four populations from North Dakota, and there was genetic differentiation both within and among locations. Genetic differentiation between the hard red spring and soft red winter wheat production regions was moderate (F_{ST} = 0.168), but whether this is due to differences in wheat production or to geographical variation cannot be determined. These results suggest that sexual reproduction occurs in *P. nodorum* populations in the major wheat-growing regions of the central United States, and that geographically separated populations can be genetically differentiated, reflecting either restrictions on gene flow or selection.

Additional keywords: genetic diversity, population genetics, multilocus analysis, *Septoria nodorum*.

Phaeosphaeria nodorum (E. Müller) (anamorph = *Stagonospora nodorum* [Berk.] Castellani and Germano, syn. *Septoria nodorum*) causes Stagonospora nodorum blotch (SNB) disease on wheat (*Triticum aestivum* L.) worldwide (15,16). In the United States, *P. nodorum* is widely distributed in humid, temperate climates (25,38). The fungus produces host-selective toxins (18) and induces typical lens-shaped necrotic and chlorotic lesions in susceptible or sensitive wheat (15,18). Consequently, it reduces both grain quality and yield (16). Sexual spores (ascospores) are released from pseudothecia in infected plant tissues to initiate SNB epidemics in the field (2,9). Previous studies reported that asexual spores (pycnidiospores) are dispersed locally by rain splash (37), while ascospores are wind dispersed and potentially can be blown over long distances (7,23,24). Strategies for managing SNB include cultural practices such as crop rotation, stubble management, fungicides, and the use of disease-resistant cultivars.

The genetic structure of *P. nodorum* populations has been analyzed at both regional and continental scales using random amplified polymorphic DNA (RAPD) (10), restriction fragment length polymorphism (RFLP) (23,24,27,28,31,47) and amplified fragment length polymorphism (AFLP) (3) markers. Low genetic differentiation was observed among populations of *P. nodorum* in the United States (23) and Switzerland (24), suggesting that gene flow was common. RAPD and RFLP markers have been augmented by microsatellite (simple-sequence repeat [SSR]) markers that have been used successfully to investigate genetic variation in several fungal pathogens (13,22,26,46) including species of *Septoria* (20,35). Recently, 12 SSR loci were developed (45) and used to examine migration patterns in *P. nodorum* (44).

The fungus has both asexual and sexual stages (23,24), and the two mating-type idiomorphs (*MAT1-1* and *MAT1-2*) have been characterized (5). Previous studies showed that both mating types occur at near-equal frequencies (41,42), with only a few exceptions (5,21). For example, Sommerhalder et al. (42) determined mating-type frequencies from five U.S. states and five other countries, and found a departure from a 1:1 ratio only in one location in Oregon. The small sample from North Dakota (n = 18) had the highest clonal fraction of any population analyzed, yet still had both mating types in a 1:1 ratio. However, nothing is known about mating-type frequencies of populations of *P. nodorum* from the other major wheat-growing areas of the north-central United States or from the soft red winter wheat region of Indiana and Ohio.

Previous analyses identified very little genetic differentiation among populations of *P. nodorum* (23,24), but this may not hold for those from the major wheat production regions of the central United States. The humid, milder climate of Indiana and Ohio is ideally suited for the production of soft red wheat grown as a winter crop, while the harsher conditions of North Dakota and Minnesota are better suited for the cultivation of hard red wheat planted during spring. These two market classes of wheat (hard red spring versus soft red winter) come from divergent breeding pools adapted to different climatic conditions. Infections of *P. no-*

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doi:10.1094/PHYTO-98-1-0101

dorum on winter wheat can be initiated during the fall so that it may have many more cycles of asexual reproduction compared to populations on hard red wheat planted during spring. Populations on hard red spring wheat must survive on infected stubble from late summer through fall and winter, while those on soft red wheat need to survive a host-free period only during late summer and early fall. Therefore, populations of *P. nodorum* from the north-central versus the midwestern states may be exposed to different selection pressures caused by cultivar, climate, or host-free periods in addition to the large geographical separation so may be much more likely to be genetically differentiated compared to other populations tested.

The main goal of this study was to determine the genetic structure of *P. nodorum* populations collected from Minnesota and North Dakota, and Indiana and Ohio, representing the north-central hard red spring and the midwestern soft red winter wheat production regions of the United States, respectively. The specific objectives were to (i) determine frequencies of the *MAT1-1* and *MAT1-2* alleles of *P. nodorum* and gametic disequilibrium to test for the signature of possible sexual recombination in each population; and (ii) assess the spatial distribution of genetic diversity in *P. nodorum* collected from hard red spring and soft red winter wheat to test for possible genetic differentiation between pathogen populations from these two production regions.

MATERIALS AND METHODS

Fungal populations. Most samples were collected by a random sampling strategy prior to the heading stage. Each population was defined on a location or wheat cultivar basis (Table 1). The four Indiana populations (IN1, IN2, IN3, and IN4) were collected from breeding lines or cultivars of soft red winter wheat in Tippecanoe County during 1995. Populations IN1 and IN4 were from the highly susceptible cultivars Caldwell and Monon, respectively. Population IN2 was from the susceptible breeding lines 21919, 21960, and 21968, while IN3 was from resistant lines 1196, 1656, and Roazon. The total sampling area for each population was ≈10 m^2 and the geographic distances among the fields were ≈0.1 km. In North Dakota, diseased leaves showing necrotic lesions were collected randomly from hard red spring wheat cultivars in fields located near state highways. Approximately 30 leaves were collected from each field by covering ≈500 m^2 of area. The four North Dakota populations were collected near Fargo, Devils Lake, and Langdon during the 2001 to 2004 seasons (ND1, ND2, and ND3), and from near Minot and Harvey during 1995 (ND4). In Minnesota, isolates were obtained from infected leaves of hard red spring wheat cultivars collected near Crookston (Polk Co.) and Rosemount (Dakota Co.) during 1995. Three populations (OH1, OH2, and OH3) were collected from Hancock, Henry, and Paulding Counties in northwestern Ohio during 1995. Each collection was made from naturally infected leaves of soft red winter wheat cultivars covering an area of ≈300 m^2 in each field. In total, 308 isolates of *P. nodorum* collected from the north-central and the midwestern regions of the United States were analyzed. These 12 populations were further combined into spring versus winter wheat populations to represent larger geographic areas. For this analysis, all isolates collected from hard red spring wheat cultivars in Minnesota and North Dakota were combined to represent the hard red spring wheat population (n = 121). Similarly, all isolates collected from soft red winter wheat cultivars in Indiana and Ohio were grouped to represent the soft red winter wheat population (n = 187).

Isolation of the fungus. During 2001 to 2004, infected wheat leaves showing typical symptoms of SNB were cut into 2 × 2 cm sections and placed in 9-cm-diameter plastic petri dishes containing three layers of Whatman No. 1 filter paper, with 10 leaf pieces per dish. To keep the filter papers moist, 3 ml of sterile distilled water were poured into each plate. The leaf pieces were incubated under continuous light for 48 to 72 h at 22°C to facilitate oozing of pycnidiospores out of pycnidia. Incubated leaf pieces were examined under a stereomicroscope, and pycnidiospores were collected by touching cirri from individual pycnidia with a glass needle and transferring onto V8-PDA (150 ml of V8 juice, 10 g of Difco potato dextrose agar, 3 g of calcium carbonate, 10 g of Bacto agar, and 850 ml of distilled water) plates.

During 1995, isolates were obtained similar to the procedures described above, except cirri containing pycnidiospores were washed from the leaves in sterile distilled water and plated onto water agar. Single germinated pycnidiospores were transferred to plates and incubated at room temperature. Pure cultures of the isolates were stored on lyophilized filter-paper strips at –80°C. All other isolates were stored as mycelial plugs at –20°C.

Haplotype identification. Mycelia of each isolate were grown in flasks containing 50 ml of Fries liquid medium (12) for 5 to 7 days on an orbital shaker at 120 rpm (VWR International, Inc., West Chester, PA). Genomic DNA was extracted as described previously (11). To identify haplotypes in each population, 10 RAPD (10) and 12 SSR (45) primers were evaluated for polymorphism on DNA from a panel of 10 isolates of *P. nodorum* selected randomly from the four states. PCR conditions for RAPD (10) and SSR (45) analyses were as described previously. The two RAPD primers, 9441 (5′-ACGCGCATGT-3′) and 9498 (5′-TGGAGAGCAG-3′), and the five SSR loci *SNOD1*, *SNOD3*, *SNOD5*, *SNOD8*, and *SNOD23*, revealed a high polymorphism and were used to identify haplotypes of *P. nodorum*. The five polymorphic SSR primer pairs (for loci *SNOD1*, *SNOD3*, *SNOD5*,

TABLE 1. Summary information for 308 isolates of *Phaeosphaeria nodorum* sampled from 12 populations in the north-central and midwestern regions of the United States

State	Region	Location[a]	Population	Sample size	Host cultivar	Wheat type[b]
Indiana	Midwest	West Lafayette (Tippecanoe Co.)	IN1	38	Caldwell	SRWW
			IN2	45	Breeding lines 21919, 21960 and 21968	SRWW
			IN3	33	Resistant lines 1196, 1656, and Roazon	SRWW
			IN4	24	Monon	SRWW
Minnesota	North-central	Crookston (Polk Co.) and Rosemount (Dakota Co.)	MN	10	Unknown	HRSW
North Dakota	North-central	Fargo (Cass Co.)	ND1	19	Alsen and Parshall	HRSW
		Devils Lake (Ramsey Co.)	ND2	28	Alsen and Granite	HRSW
		Langdon (Cavalier Co.)	ND3	43	Alsen	HRSW
		Harvey (Wells Co.) and Minot (Ward Co.)	ND4	21	Unknown	HRSW
Ohio	Midwest	Durkirk (Hancock Co.)	OH1	14	Unknown	SRWW
		Custar (Henry Co.)	OH2	18	Unknown	SRWW
		Latty (Paulding Co.)	OH3	15	Unknown	SRWW

[a] Nearest city and (county) of collection.
[b] SRWW = soft red winter wheat; HRSW = hard red spring wheat.

SNOD8, and *SNOD23*) were used to further examine the genetic structure of *P. nodorum* populations as described previously (45). Polymerase chain reaction (PCR)-amplified products were separated on 6% urea nondenaturing polyacrylamide gels (40).

Mating type determination. Mating type alleles in each population were determined using the four mating type primers identified previously (5). These primers amplified products of ≈360 bp for *MAT1-1* isolates and ≈510 bp for *MAT1-2*. All primers were synthesized by DNA Technologies (Integrated DNA Technologies, Inc., Coralville, IA), and multiplex PCR amplifications were performed as described previously (42). Genomic DNA extracted from a pure culture of *P. nodorum* standard isolate Sn2000 was included as a positive control in all assays. Negative control reactions contained the PCR mixtures with 2 µl of sterile distilled water without DNA template or a combination of primer solutions and water. All amplifications were confirmed by two independent PCR analyses.

Data analysis. Unless stated otherwise, clone-corrected data were used for all analyses. Allele frequencies at each SSR locus were estimated from each population using POPGENE (PC version 1.32, F. C. Yeh, R. C. Yang, and T. J. B. Boyle, Molecular Biology and Biotechnology Center, University of Alberta, Canada). Nei's unbiased gene diversity was used to estimate genetic diversity in the total population (H_T) and within populations (H_S) (32). Genotype diversity (D) for each population was calculated using MultiLocus version 1.3 (1) as $D = (n/n - 1)(1 - \Sigma p_i^2)$, where p_i is the frequency of the ith genotype and n is the number of individuals sampled, which is the probability that two individuals taken at random have unique genotypes (1). Two approaches were applied to clone-corrected data to test whether the observed pattern of genetic variation was consistent with the null hypothesis of sexual reproduction. First, significant departures from the expected 1:1 ratio in mating-type frequencies were tested with a χ^2 test (14). Second, the index of association (I_A) (1,8) was used to analyze gametic disequilibrium. To test for departure from gametic equilibrium, locus pairs were considered to be in gametic disequilibrium if the exact probability was less than 0.05.

Nei's unbiased measures of genetic identity (I), and genetic differentiation (F_{ST}) were estimated to compare between population pairs (32). The MN population had only nine isolates after clone correction so that this population was excluded from these analyses. The F_{ST} values were analyzed by GenAlEx V5 software (34). The significance levels ($P < 0.001$ or 0.05) were tested using 1,000 permutations. These analyses were repeated at the levels of regions (states) and of spring versus winter wheat. Regions were made by pooling isolates from each state into single populations from IN, OH, and ND (MN was excluded from this analysis due to small sample size), and by pooling isolates from soft red winter wheat in IN and OH versus those from hard red spring wheat in MN and ND. Allele frequencies at each SSR locus, Nei's gene diversity (32), and Nei's unbiased measures of genetic identity and genetic distance (33) were calculated for the pooled regional populations and for those from the hard red spring versus soft red winter wheat production areas.

RESULTS

Mating type distribution. Among 293 isolates analyzed in the clone-corrected data set, 143 were *MAT1-1* and 150 were *MAT1-2* (Table 2). Frequencies of the two mating types did not differ significantly from a 1:1 ratio either by state (Table 2) or by individual population sampled (Table 3). Three isolates produced the

TABLE 2. Distribution and segregation of mating type alleles of *Phaeosphaeria nodorum* in the north-central and midwestern regions of the United States

Population	Sample size[a]	Clone-corrected sample size[b]	*MAT1–1*	*MAT1–2*	χ^2	P[c]
Indiana	140	133	63	70	0.270	0.597
Minnesota	10	9	5	4	0.110	0.738
North Dakota	111	109	53	56	0.037	0.842
Ohio	47	42	22	20	0.238	0.888
Total	308	293	143	150	0.218	0.639

[a] Total number of isolates obtained before clone correction.
[b] Combined results from two random amplified polymorphic DNA primers (9441 and 9498) (10) and five microsatellite loci (*SNOD1*, *SNOD3*, *SNOD5*, *SNOD8*, and *SNOD23*) (45) were used to identify and remove duplicate clones from each population.
[c] Probability that the χ^2 value differs from an expected ratio of 1:1 with 1 degree of freedom.

TABLE 3. Genetic diversity of *Phaeosphaeria nodorum* populations sampled from the north-central and midwestern regions of the United States

Population	Sample size	Clone-corrected	Mating type		Gene diversity (H)[b]	Genotype diversity (D)[c]	Index of association (I_A)[d]
			Ratio[a]	χ^2			
IN1	38	35	15:20	0.714	0.289	0.867	0.303
IN2	45	44	18:26	1.455	0.194	0.713	0.030
IN3	33	31	16:15	0.032	0.268	0.781	0.222
IN4	24	23	14:9	1.087	0.229	0.731	–0.524
MN	10	9	5:4	0.111	0.316	0.778	nc[e]
ND1	19	18	7:11	0.889	0.380	0.758	1.734**
ND2	28	28	10:18	2.286	0.293	0.775	0.339*
ND3	43	42	25:17	1.524	0.519	0.972	0.288**
ND4	21	21	11:10	0.048	0.408	0.886	0.061
OH1	14	12	6:6	0.000	0.411	0.954	–0.129
OH2	18	16	9:7	0.250	0.175	0.600	–0.022
OH3	15	14	9:5	1.143	0.180	0.758	–0.066

[a] *MAT-1-1*:*MAT1-2*. None deviated significantly from the ratio of 1:1 expected assuming random mating.
[b] Gene diversity (32) within populations was calculated from clone-corrected data based on five microsatellite loci (*SNOD1*, *SNOD3*, *SNOD5*, *SNOD8*, and *SNOD23*).
[c] Genotype diversity within populations was calculated as $(n/n - 1)(1 - \Sigma p_i^2)$ from the clone-corrected data, where p_i is the frequency of the ith genotype and n is the number of individuals sampled, which is the probability that two individuals taken at random have unique genotypes (1).
[d] The index of association (I_A) statistic was estimated for each population (8). The significance of I_A was tested with 1,000 randomizations of the data by comparing the observed value to that expected under the null hypothesis of $I_A = 0$ (1). The null hypothesis of multilocus linkage equilibrium was rejected if $P <$ 0.001 or 0.05. ** Significant at $P = 0.001$; * significant at $P < 0.05$.
[e] nc = not calculated due to small sample size.

bands expected for both *MAT1-1* and *MAT1-2*, and DNA from five other isolates failed to amplify with the mating-type primers. Therefore, these 8 isolates were excluded from further analyses.

Genetic diversity within populations. The five SSR loci generated 14 alleles among the 12 populations of *P. nodorum* (data not shown). Across all five loci, the ND1, ND2, ND3, and ND4 populations had the most even allele frequencies, while the MN population had the fewest alleles. Although allele frequencies differed, most populations of *P. nodorum* contained the same 2 to 3 alleles per locus with an average of 2.8. Allele sizes for each locus ranged from 252 to 552 bp. The highest numbers of alleles were found in the ND2, ND3, and ND4 populations. *H* was moderate to high for the 12 populations, and ranged from 0.175 in OH2 to 0.519 in ND3 (Table 3). Among 308 *P. nodorum* isolates analyzed, 293 distinct haplotypes were found (Table 2), most of which were detected only once in each population. Identical haplotypes usually were found among isolates originating from the same leaf, or sometimes from different leaves from the same field. Total genotype diversity for the whole sample was $D = 0.907$. The ND3 population had the highest $D = 0.972$, while the OH2 population had the lowest D of 0.600 (Table 3).

Gametic disequilibrium. Estimates of I_A for the ND2 population differed significantly from zero ($P < 0.05$) (Table 3), while those for populations ND1 and ND3 were highly significant ($P < 0.01$). Eight of the 11 populations (IN1, IN2, IN3, IN4, ND4, OH1, OH2, and OH3) showed no evidence of gametic disequilibrium.

Genetic identity (*I*) and genetic differentiation (F_{ST}). Pairwise comparisons of *I* values across *P. nodorum* populations were generally high (Table 4). The lowest *I* value was observed between the IN3 and ND2 population pair (0.499). High *I* values were observed in pairings between the four IN populations, between the four IN populations and Ohio populations OH2 and OH3, between OH2 and OH3 directly, and between the North Dakota populations ND2 and ND4. The highest *I* of 0.993 was between populations IN2 and OH2. The mean F_{ST} over all pairwise comparisons was moderate (0.288). The lowest F_{ST} was 0.003, between the population pair IN4-OH2 (Table 4). The highest F_{ST} value was 0.604, between populations IN4 and ND2. The F_{ST} value for the two most geographically distant populations, ND4 and OH1, was 0.304. Low to moderate F_{ST} values were observed between populations within the midwestern (Indiana and Ohio) wheat production region. In comparisons between populations within this region, F_{ST} values ranged from 0.003 (IN4 and OH2) to 0.328 (IN4 and OH1). Differentiation between population pairs within the geographically close populations from Indiana generally was very low, with F_{ST} ranging from 0.005 (IN3-IN4) to 0.102 (IN2-IN3) (Table 4). The corresponding values were higher in Ohio, where F_{ST} ranged from 0.165 (OH2 and OH3) to 0.335 (OH1 and OH2). Differentiation between population pairs in ND ranged from very low ($F_{ST} = 0.044$ between ND2 and ND4) to quite high (0.513 between ND1 and ND2).

Gene diversity was almost twice as high as in the isolates sampled from spring wheat ($H_S = 0.503$) compared to those from winter wheat ($H_S = 0.269$) (Table 5). Nei's genetic distance and genetic identity between the populations from spring versus winter wheat cultivars were 0.168 and 0.765, respectively, indicating a moderate degree of genetic differentiation. Most of this variation was due to differences between Indiana and North Dakota. The F_{ST} between all populations in Indiana versus those in Ohio was only 0.046, while the differentiation between Indiana and North Dakota was 0.338 and between North Dakota and Ohio it was 0.261.

Gene diversity was similar among all loci within each region (Table 5). The exception was *SNOD8*, which was much less diverse than the other loci among the isolates from winter wheat. Genetic differentiation varied dramatically among the loci, from a low F_{ST} of 0.038 for loci *SNOD3* and *SNOD23* to a maximum of 0.462 for locus *SNOD8* (Table 5).

DISCUSSION

Populations of *P. nodorum* in the hard red spring wheat region of Minnesota and North Dakota are exposed to differences in climate, cropping system, cultivars of wheat, and length of host-free period compared to those in the soft red winter wheat region that includes Indiana and Ohio. Variation in selection imposed by these variables could cause the populations to become differen-

TABLE 5. Genetic diversity and differentiation by locus in *Phaeosphaeria nodorum* populations sampled from spring (North Dakota and Minnesota) versus winter (Indiana and Ohio) wheat production areas of the United States

Microsatellite locus	Gene diversity[a] Spring wheat ($n = 121$)	Winter wheat ($n = 187$)	F_{ST}
SNOD1	0.593	0.249	0.073
SNOD3	0.402	0.243	0.038
SNOD5	0.522	0.336	0.208
SNOD8	0.443	0.062	0.462
SNOD23	0.555	0.452	0.038
Mean	0.503	0.269	0.168

[a] According to Nei (32).

TABLE 4. Pairwise comparisons of Nei's unbiased measure of gene identity (above diagonal) and genetic differentiation (F_{ST}) (below diagonal) in 11 populations of *Phaeosphaeria nodorum* sampled from the north-central and midwestern regions of the United States[a]

Population	IN1	IN2	IN3	IN4	ND1	ND2	ND3	ND4	OH1	OH2	OH3
IN1	…	0.964[b]	0.989	0.976	0.810	0.556	0.649	0.622	0.786	0.974	0.925
IN2	0.081*[c]	…	0.959	0.971	0.806	0.616	0.665	0.682	0.840	0.993	0.961
IN3	0.078*	0.102**	…	0.987	0.838	0.499	0.590	0.589	0.808	0.976	0.923
IN4	0.035 ns	0.078*	0.005 ns	…	0.806	0.536	0.606	0.608	0.813	0.988	0.915
ND1	0.231**	0.340**	0.233**	0.305**	…	0.502	0.664	0.658	0.816	0.806	0.753
ND2	0.507**	0.570**	0.580**	0.604**	0.513**	…	0.889	0.966	0.621	0.582	0.553
ND3	0.302**	0.373**	0.361**	0.370**	0.247**	0.141**	…	0.889	0.731	0.628	0.597
ND4	0.413**	0.478**	0.476**	0.504**	0.347**	0.044 ns	0.100*	…	0.735	0.656	0.627
OH1	0.268**	0.319**	0.286**	0.328**	0.192*	0.473**	0.208**	0.304**	…	0.826	0.798
OH2	0.044 ns	0.040 ns	0.042 ns	0.003 ns	0.318**	0.602**	0.359**	0.487**	0.335**	…	0.943
OH3	0.124*	0.098*	0.155*	0.213**	0.331**	0.582**	0.347**	0.465**	0.317**	0.165*	…

[a] The Indiana populations are IN1 = Caldwell, IN2 = breeding lines 21919, 21960, and 21968, IN3 = resistant lines 1196, 1656, and Roazon, and IN4 = Monon; the North Dakota populations are ND1 = Fargo, ND2 = Devils Lake, ND3 = Langdon, and ND4 = Minot; and the Ohio populations include OH1 = Hancock, OH2 = Henry, and OH3 = Paulding counties.

[b] Measures of Nei's gene identity calculated in each population (32). A maximum identity of 1.0 occurs when the same alleles occur at identical frequencies for a pair of populations.

[c] Genetic differentiation (F_{ST}) was calculated to compare population pairs (32). ** Significant at $P = 0.001$; * significant at $P < 0.05$; and ns = not significant.

tiated genetically. In addition to environmental and cultivar differences, the closest sampled populations in Indiana and Minnesota are separated by more than 800 km, which may impose a barrier to migration. This distance is too large for dispersal of ascospores (39), and opportunities for seedborne migration (3,36) are minimized due to lack of mixing between these two market classes of wheat. For these reasons it seems reasonable that populations of *P. nodorum* could differ significantly between the hard red spring versus soft red winter production regions of wheat in the north-central and midwestern United States.

In addition to possible genetic differentiation between the regions, differences in cropping system and host-free periods could affect the clonal structure of *P. nodorum* populations in each wheat production area. Infections of winter wheat can occur soon after emergence of seedlings and might allow for extra cycles of asexual reproduction during fall and early spring compared to populations of *P. nodorum* on spring-planted wheat. The much shorter host-free period in Indiana and Ohio (about three months from mid-July to mid-October) might permit greater survival of clones from season to season on infested stubble near freshly planted wheat crops, for spread by splash dispersal of pycnidiospores. Overwinter survival in Minnesota and North Dakota requires seven or more months from the middle of September until May. This could decrease survival overall and may increase the frequency of epidemics initiated by windblown ascospores from distant locations. Contributions of seedborne inoculum are not known but presumably would be similar in each region. For these reasons, we might expect to see a much greater survival and reproduction of clonal genotypes in the soft red winter region represented by Indiana and Ohio compared to the hard red spring wheat region of Minnesota and North Dakota. However, very few clones were found in both regions, with identical haplotypes only identified on isolates from the same leaf or occasionally on different leaves from the same field. Although clonal reproduction was detected and certainly is important for spreading the disease over short distances, it does not seem to be the predominant mode of reproduction in these populations, at least at the spatial scales sampled.

Instead, the high genotypic diversity and near-equal frequency of mating types in each population are consistent with the hypothesis that ascospores play an important role in initiating and maintaining epidemics of *P. nodorum* in the north-central and midwestern United States. This result agrees with previous reports of equal mating-type frequencies in populations of *P. nodorum* worldwide (41,42). Exceptions have been rare, and usually showed an excess of *MAT1-1* over *MAT1-2*. For example, Sommerhalder et al. (42) found a barely significant excess of *MAT1-1* in only one out of 18 fields tested worldwide; all other fields had equal frequencies of both mating types. Halama (21) reported a significant excess of *MAT1-1* over *MAT1-2* among 101 isolates tested from a global sample by mating-type tests in the laboratory. Interestingly, approximately 40% of those isolates could not be assigned to either mating type due to failure of the tests. Whether these isolates are sterile or simply require different conditions to initiate mating is not known, but if they all turned out to be *MAT1-2* then the ratio with *MAT1-1* would not have differed significantly from 1:1. Both *MAT1-1* and *MAT1-2* were present in isolates of *P. nodorum* collected from Kazakhstan and Russia, but only *MAT1-1* was found in a limited sample of seven isolates from Tajikistan (48). Whether this reflects a biological difference or merely a too-small sample in Tajikistan is unclear. Bennett et al. (5) analyzed two field populations of *P. nodorum* from New York, and found a high frequency of *MAT1-1* among 22 isolates from one field, but equal numbers of both mating types among 32 isolates in another field. We found no evidence for an excess of *MAT1-1* in any of the *P. nodorum* populations sampled from the north-central and midwestern regions of the United States. Based on these findings, plus the analysis of SSR loci that revealed high levels of genotypic diversity in all *P. nodorum* populations sampled, sexual recombination probably occurs frequently within naturally infected field populations in the central United States. This result is consistent with previous analyses of multilocus associations of RFLP markers and frequencies of mating type alleles (23,24,28,42), and is not surprising, because ascocarps of *P. nodorum* have been observed in the eastern soft red winter wheat region of the United States (9) and most likely occur in the north-central and midwestern regions. However, our analysis did not address the question of seedborne inoculum, which can contribute significantly to epidemics in some locations (4), including populations with high levels of genotypic diversity (3). Although sexual reproduction and ascospores certainly appear to play an important role, their contribution relative to seedborne inoculum in the north-central and midwestern United States remains unknown and should be estimated for a complete picture of the epidemiology of the disease in this region.

The multiplex-PCR mating type assay occasionally gave inconclusive results in which both, or neither, of the expected bands amplified. In this study, three isolates produced both *MAT1-1* and *MAT1-2* amplicons, possibly due to mixed cultures or slight contamination of DNA samples. DNA of five isolates gave no amplification with the mating-type primers, most likely due to sensitivity of the multiplex assay to PCR conditions or to impurities in the samples. These eight isolates represented about 2.6% of the entire sample. Similar problems occurred in about 10% of 1,207 isolates of *P. nodorum* analyzed previously with the same primers (42). Two intriguing possible explanations for aberrant mating-type results other than contamination or amplification failure are that isolates with both mating-type products could be homothallic, and lack of amplification could reflect mutations in the parts of the genome complementary to the primer sequences or possibly even deletion of part or all of the mating-type locus. Deletion of the mating-type locus could explain the apparent sterility observed by Halama (21). However, hypotheses about homothallism and sterility must be tested by additional experimentation.

One of the most important findings of this study was the detection of significant genetic differentiation among populations. The genetic differentiation value estimated for *P. nodorum* in the central United States (mean F_{ST} over all pairwise comparisons = 0.288) was considerably higher than those for *P. nodorum* and other species of *Septoria* reported previously (17,23,49). For example, Keller et al. (23) found an F_{ST} value of 0.004 among one Swiss and two American populations of *P. nodorum*, and similarly low values have been reported among populations worldwide (44). Other *Septoria* fungi in North America also had lower values, such as *Septoria tritici* with a differentiation value of 0.08 (49), and *S. musiva* with an overall value of 0.20 (17). The low differentiation observed among those fungal populations was thought to be due to high migration facilitated by wind-dispersed ascospores and other means of gene flow (6,23). A pathogen with similar levels of genetic differentiation to those we found among populations of *P. nodorum* was the chestnut blight fungus, *Cryphonectria parasitica*, for which moderate to high levels of differentiation (G_{ST} = 0.20 to 0.31) were observed among 13 subpopulations in eastern North America (29). Differentiation in this case was thought to be the result of genetic drift, which occurred due to possible founder effects or cyclical reductions in effective population size coupled with limited gene flow (29,30). Our F_{ST} estimates for *P. nodorum* confirmed that differentiation occurred between populations from spring cultivars in the north-central hard red wheat region compared to those from soft red winter wheat in the midwestern region. The level of genetic differentiation observed in this study (F_{ST} = 0.168) probably indicates that there are some restrictions to gene flow between populations on the hard red spring versus soft red winter market classes of wheat.

Another difference between the results of this study and those published previously for *P. nodorum* was the identification of significant ($P < 0.05$) gametic disequilibrium in three of the four North Dakota populations analyzed. Previous reports found populations worldwide to be in equilibrium (3,44). The causes of disequilibrium in the North Dakota populations are not known, but could include selection, migration of individuals or admixture of populations, asexual reproduction, or physical linkage of the markers on chromosomes. Asexual reproduction can be eliminated because all analyses were performed on clone-corrected data sets to remove any bias caused by differential reproduction and recovery of clones. To test for physical linkage, we performed BLAST searches of the microsatellite primer sequences against the genomic sequence of *P. nodorum*. The search results revealed that loci *SNOD1*, *SNOD3*, *SNOD5*, *SNOD8*, and *SNOD23* were each single copy and were located on supercontigs 1, 5, 28, 4, and 8, respectively, so could not reflect gametic disequilibrium caused by linkage.

That leaves selection, admixture of populations, or possibly sampling phenomena as the most likely explanations for the observed gametic disequilibrium. All of the microsatellites came from expressed sequence tag (EST) sequences and the GCA repeat of *SNOD3* was in the coding region, so selection, either directly on the microsatellites or by hitchhiking with the linked coding regions, remains a possibility. The annotations provided for these loci revealed nothing of interest for *SNOD1*, while *SNOD3* had similarity to a GTPase activating protein and *SNOD5* contained several putative RNA recognition sequences. The hypothetical protein coded by *SNOD8* was highly conserved in other fungi and contains a possible cell wall surface anchor according to BLAST similarities (data not shown). Although these results are somewhat intriguing, they provide no evidence for or against selection. *SNOD23* was the most interesting locus because it has similarity to ABC transporters. In *Mycosphaerella graminicola*, ABC transporter MgAtr4 can influence virulence (43), and a similar role for *SNOD23* in *P. nodorum* is possible. Therefore, selection may be an explanation for some of the patterns of genetic diversity observed among populations of *P. nodorum* in the central United States, although it must be confirmed by additional experimentation.

Admixture of populations with different allele frequencies also can cause disequilibrium. This could occur either by migration or by artificially lumping isolates from different subpopulations into a single population. The latter is a real possibility for two of the three populations that showed significant gametic disequilibrium: populations ND1 and ND2 contained isolates from two cultivars, one of which in each case was the highly susceptible Alsen (19). Although the sampled fields for each population were in close proximity, it is possible that there were differences in allele frequencies among the samples that could have generated a signal of disequilibrium once they were combined. However, population ND3 was from a single field so admixture is not a likely explanation for the disequilibrium observed in that sample.

A final possible cause of disequilibrium could be sampling phenomena, particularly involving small samples or rare alleles. No rare alleles occurred in population ND1, but populations ND2 and ND3 had 4 and 2 alleles, respectively, that were present at frequencies of less than 0.05 and these possibly could have led to spurious disequilibrium. Sample sizes were not huge but were sufficient; populations showing significant gametic disequilibrium had sample sizes from 19 to 43. Additional sampling of populations of *P. nodorum* in North Dakota is warranted to confirm whether disequilibrium occurs commonly and to determine its cause.

ACKNOWLEDGMENTS

We thank D. Long and G. Zhang for collecting infected leaf material and making isolations, respectively, H. Hayden for data analysis, and B. P. Chhetri and J. Cavaletto for technical assistance. G. Secor and two anonymous reviewers made helpful comments on a previous draft of the manuscript. This work was supported in part by the Agricultural Experiment Station of North Dakota State University and USDA CRIS project 3602-22000-013-00D.

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