

Frequency of *Phaeosphaeria nodorum*, the Sexual Stage of *Stagonospora nodorum*, on Winter Wheat in North Carolina

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Accepted for publication 25 March 2006.

ABSTRACT

Cowger, C., and Silva-Rojas, H. V. 2006. Frequency of *Phaeosphaeria nodorum*, the sexual stage of *Stagonospora nodorum*, on winter wheat in North Carolina. *Phytopathology* 96:860-866.

Ascocarps of *Phaeosphaeria nodorum*, which causes *Stagonospora nodorum* blotch (SNB) of wheat, have not been found by others in the eastern United States despite extensive searches. We sampled tissues from living wheat plants or wheat debris in Kinston, NC, each month except June from May to October 2003. Additional wheat samples were gathered in Kinston, Salisbury, and Plymouth, NC, in 2004 and 2005. For the 3 years, in all, 2,781 fruiting bodies were dissected from the wheat tissues and examined microscopically. Fruiting bodies were tallied as *P. nodorum* pycnidia or ascocarps, “unknown” (not containing spores, potentially *P. nodorum* or other fungi), or “other fungi.” In the 2003 sample, ascocarps of *P. nodorum* were present each month after May at a frequency of 0.8 to 5.4%, and comprised a significantly higher percentage of fruiting

bodies from wheat spikes than of those from lower stems and leaves. Ascocarps also were found at frequencies <10% in some wheat debris samples from 2004 and 2005. Analysis of the nucleotide sequences of internally transcribed spacer regions of 18 genetically distinct North Carolina isolates from 2003 suggested that all were *P. nodorum*, not the morphologically similar *P. avenaria* f. sp. *triticea*. Neither the 18 isolates from 2003 nor a set of 77 isolates derived from 2004 Kinston leaf samples gave reason to suspect a mating-type imbalance in the larger *P. nodorum* population ($P \geq 0.4$). We conclude that, in North Carolina, sexual reproduction plays a role in initiation of SNB epidemics and the creation of adaptively useful genetic variability, although its relative importance in structuring this population is uncertain.

Additional keywords: *Leptosphaeria nodorum*, pseudothecia, *Septoria nodorum*.

Phaeosphaeria nodorum (E. Müller) Hedjaroude, syn. *Leptosphaeria nodorum* E. Müller (anamorph = *Stagonospora nodorum* (Berk.) Castellani & E. G. Germano, syn. *Septoria nodorum* (Berk.)) is the causal agent of *Stagonospora nodorum* blotch (SNB) of wheat, a widespread and yield-reducing disease in wheat-growing regions with relatively high rainfall.

P. nodorum is a bipolar, heterothallic fungus (19). The teleomorph has been found in many parts of the world, including Brazil (31), France (34), Germany (32), Ireland (33), Italy (16), New Zealand (36), and South Africa (23). However, in some parts of the United States, the search for ascocarps of *P. nodorum*, which are pseudothecia, has proven elusive. Researchers in Montana (37) and Oregon (8) have observed abundant *P. nodorum* ascocarps. But researchers in the northeastern (39) and southeastern (9) United States, where disease levels are often substantial, have not found *P. nodorum* ascocarps or ascospores despite intensive searches over many years.

Controversy has existed over the degree to which sexual reproduction affects the *P. nodorum* population structure. Populations collected in 1984 and 1985 from small areas of wheat fields in the United Kingdom revealed a spatially fine-grained polymorphism for electrophoretic karyotype consistent with a prominent role for ascospores in initiating infections (6). Molecular tests of gene and genotype diversity and gametic equilibrium have supported the

hypotheses that epidemics are initiated by ascospores and pathogen populations are random-mating in Texas, Oregon, and Switzerland (21,22,29).

However, Shah et al. (39) have argued that such results also could be produced by genetically heterogeneous seedborne fungal populations. These researchers have shown that the percentage of artificially infected seed is correlated with later disease incidence and severity in plots in New York state sown with that seed (39), and that wheat cultivars differ in relative susceptibility to seed infection (38). They suggest that, in at least some production systems, such as that in New York, primary inoculum for epidemics may be mainly seedborne (38).

Mating-type ratios have been studied to obtain evidence on the extent of sexual reproduction by *P. nodorum*. Among 56 *P. nodorum* isolates from Europe, Australia, the United States, Morocco, and Israel that could be assigned a mating type, Halama (18) found that the MAT1-1 allele was present in 92% of isolates and the MAT1-2 allele in the other 8%. Such an imbalance could suggest a departure from random mating in the populations giving rise to those isolates. Bennett et al. (3) sampled two fields in New York state, and found a predominance of MAT1-1 among 22 isolates from one field, but approximately equal numbers of both mating types among 31 isolates from the other field. Sommerhalder et al. (41) determined mating-type ratios of samples from five U.S. states, including two in the eastern soft red winter wheat region (Arkansas and New York), as well as five other countries. They found a departure from a 1:1 ratio in only one location (Oregon).

P. nodorum is an important pathogen of soft red winter wheat in North Carolina, although SNB epidemics are significant in fewer than 1 of 2 years (14). The purpose of this study was to

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DOI: 10.1094/PHYTO-96-0860

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determine whether the teleomorph was present in North Carolina, and at what frequency. Due to morphological and symptom similarities, *P. nodorum* can be confused with *P. avenaria* (G. F. Weber) O. Eriksson f. sp. *triticea* T. Johnson (25,26,44), the cause of Stagonospora avenae blotch of cereals. Therefore, we also conducted molecular tests on a sample of *Phaeosphaeria* isolates from North Carolina to determine their species identity.

MATERIALS AND METHODS

Sample collection. In 2003, a moderately severe SNB epidemic occurred in Kinston, NC. Starting in May, wheat debris were sampled monthly (except June) for a 6-month period from an experimental field of multiple soft red winter wheat genotypes at the Cunningham Research and Extension Center in Kinston. In May, tissue samples were collected from the susceptible cultivar USG 3209 (PVP 200100127) and from a moderately resistant NC experimental line (NC98-24182). By July, however, the post-harvest stubble was scattered among half-grown soybean plants, and it was no longer possible to identify wheat genotypes. Debris was collected from a wide enough area to include multiple host genotypes.

The collected tissues were separated into “basal” and “upper.” Basal tissues were lower leaves (F-4 and F-5) and the lower 10 cm of the stems. Upper tissues were spikes (glume, rachis, and peduncles), except in May, when flag leaves were added to spikes in order to have sufficient lesioned tissue. A limited amount of tissue was available in September 2003, leading to smaller sample sizes in that month, and no spikes were collected in October 2003.

More temporally limited collections of wheat debris were made in 2004 and 2005 in an effort to confirm the results from 2003. Samples were gathered from Kinston (12 August and 23 October 2004 and 16 June and 20 September 2005), Salisbury (17 June and 11 August 2004), and Plymouth (15 June 2004), NC.

For comparison, fruiting bodies also were examined from small wheat leaf samples that had been collected for a different experiment and, thus, were available only for certain dates and canopy positions. From Griffin, GA, 100 fruiting bodies were examined from upper leaf samples collected in the Lineas Avanzadas del Cono Sur (LACOS) nursery on 17 April 2003, and 179 fruiting bodies were examined from F-1 leaves collected from cv. NK Coker 9668 on 2 June 2003. Those collections were performed by B. Cunfer (University of Georgia). In addition, 78 fruiting bodies were examined from flag or F-1 leaves of cv. Elkhart collected by E. DeWolf (Penn St. University) near State College, PA, on 11 July 2003.

Fruiting body examination. In the laboratory, wheat tissue containing apparent lesions or individual fruiting bodies of *P. nodorum* was selected and broken into pieces 0.5 to 2 cm long. The tissue was frozen for ≈ 90 s in liquid nitrogen and ground to a coarse powder with a mortar and pestle. The powder was transferred to a microscope slide. Fruiting bodies were teased from it with a stab under a dissecting microscope and transferred into lactophenol cotton blue stain on another slide. They were squashed gently beneath a cover slip and allowed to absorb stain overnight. On the following day, identifications were performed on a light microscope with a $\times 40$ objective. This procedure was conducted either twice or three times for each tissue type collected in each month, such that an approximately equal number of fruiting bodies was examined for each tissue-month combination.

Pycnidia and ascocarps of *P. nodorum* were identified according to Eyal et al. (12) and Sutton and Waterston (42), and were classified as such only if they contained spores. Other fruiting bodies were tallied as either “unknown” if they contained no spores and, thus, could be immature or discharged fruiting bodies of *P. nodorum* or another fungus; or “other fungi” if they contained asci and ascospores or pycnidiospores of fungi other than *P. nodorum*.

A two-way χ^2 test was performed to determine whether, taking only *P. nodorum* fruiting bodies into account, the percentages of ascocarps and pycnidia found in a given month of 2003 were significantly different on basal versus upper tissues. Due to small sample sizes, Fisher’s exact test was used for the month of September.

Ninety-five percent binomial confidence limits (BCLs) were calculated for frequencies of *P. nodorum* ascocarps and pycnidia, in each case using the proportion of that fruiting body type in contrast to all other types in the sample. PROC FREQ of SAS (SAS Institute, Cary, NC) was used to calculate asymptotic and exact confidence limits; asymptotic limits were reported except where counts were 0, when exact limits were reported.

Species identification. For molecular evidence on the species of *Phaeosphaeria* present, a sample population of 26 isolates was created (STAN1 to STAN26). These isolates were derived from leaves sampled in North Carolina in May 2003, but were not the same genetic individuals as those that produced fruiting bodies classified in this study. In all, 21 isolates were obtained from flag or F-1 leaves of cvs. USG 3209 and Saluda (PI 480474) sampled randomly in the Kinston field in which the fruiting bodies were collected, and 5 isolates were derived from F-1 leaves of cv. NK Coker 9663 (PI 596345) sampled in an experimental field in Plymouth, NC. To make these isolations, leaf segments containing pycnidia were affixed to microscope slides and placed in moist chambers overnight. The cirrhi were transferred individually to potato dextrose agar amended with kanamycin at 50 mg/liter. Monopycnidial cultures were stored on yeast-malt agar slants at 4°C.

Isolates were grown on a rotary shaker for 7 days in yeast-sucrose broth (4 g of yeast extract and 4 g of sucrose per liter of water) amended with gentamicin at 50 mg/liter. Mycelium was harvested by filtration through cheesecloth, frozen to -80°C , and lyophilized. DNA was extracted using Qiagen DNeasy Plant Mini Kit (QIAGEN Sciences, MD) according to the manufacturer’s protocol, and quantified with a spectrophotometer.

The internal transcribed spacer (ITS) region of each isolate was amplified with primers ITS5 and ITS4 (47). The amplicons included the ITS1, ITS2, and 5.8S sequences, and portions of the 18S and 28S sequences of the nuclear ribosomal DNA. The reaction mixture was optimized by using 5 \times Buffer (Q-Biogene, Inc., Carlsbad, CA) diluted to 1 \times and containing *Taq* DNA polymerase at 0.02 U/ μl ; dNTPs, each 100 μM ; Tris-HCl, pH9.0, 10 mM; KCl, 50 mM; MgCl₂, 1.5 mM; Triton X-100, 0.1%; bovine serum albumen at 0.2 mg/ml; glycerol, 1.8%; and 100 ng of DNA in 25 μl of total volume. Amplifications were performed with a PTC-100 thermocycler Programmable Thermal Controller (MJ Research, Inc., Watertown, MA) with the following cycling parameters: 95°C for 4 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension for 10 min at 72°C. The size and quantity of amplification products were verified on a 1.2% agarose gel, stained with EtBr, and visualized using a UV trans-illuminator AlphaImager 2200 Imaging Systems (Alpha Innotech Corp., San Leandro, CA).

The amplified products were purified with the QIAquick spin columns polymerase chain reaction (PCR) Purification Kit (Qiagen Inc., Chatsworth, CA). Both strands were direct-sequenced from the PCR products with the Beckman CEQ 8000 Genetic Analysis System (BioServe Biotechnologies Ltd., Bethesda, MD).

Sequences of amplified products of the North Carolina isolates were compared with the following GenBank accessions: *P. nodorum* isolates U77362 (45), AF321323, and AF181710 (17); *P. avenaria* isolates AF439472 (5) and AY196988; *P. arenaria* isolate U77360 (45); *P. nigrans* isolate AF439492 (5); and *Mycosphaerella graminicola* isolate U77363 (45).

The consensus sequence for each isolate was determined using Bioedit (version 5.0.9; Department of Microbiology, North Carolina State University). DNA sequences were aligned using the profile alignment mode of Clustal W, version 1.81 (43), and were

edited manually only for 18S and 28S sequences. Separate analyses were performed on ITS1, ITS2, and the entire ITS region, including the 5.8 rRNA gene. All sequences were trimmed because sequences downloaded from GenBank were of different lengths, and only bases 58 to 565 of the putative *P. nodorum* sequences were used for the entire ITS analysis.

The percentage of similarity between the ITS sequence of each isolate in the North Carolina sample and those of *P. nodorum* accessions in GenBank was determined using BLAST (1). Genetic distances among all isolates were calculated by the neighbor-joining method of Saitou and Nei (35), using Kimura's two-parameter substitution model for estimating evolutionary distances (24). Phylogenetic trees were constructed with MEGA 3.1 software (27), and confidence in the trees was assessed using a bootstrap test with resampling of 5,000 replicates (13).

Clone correction and mating-type determination. The 26-isolate population from 2003 was clone corrected, because multiple isolates had originated from the same leaf and, thus, were more likely to be clones. Clone correction is a procedure in which duplicate isolates with a given DNA fingerprint or multilocus haplotype are purged from a sample either altogether or according to sampling criteria (7). Clone correction was performed in this case because these isolates were to be used for molecular species identification and mating-type identification, and multiply sampling the same individual would lead to potential bias in conclusions about frequencies of mating types or *Phaeosphaeria* spp. Three kinds of PCR were used to create DNA fingerprints: dispersed repetitive elements (rep-PCR), microsatellite-primed (MP-PCR), and random amplified polymorphic DNA (RAPD) PCR. The rep-PCR primer sets corresponded to the BOX, ERIC, and REP repetitive elements (10,30,46). The MP microsatellite motifs (AAC)₇ and (AAG)₇ and the RAPD primers 9441, 2485, 9487, and 9542 were described by Czembor and Arseniuk (10). Amplification conditions were as in Czembor and Arseniuk (10), except that amplicons were separated and visualized on 2% agarose gels. Weak bands were not scored. Two isolates were considered to have different genotypes if they differed by at least one amplicon in rep- or RAPD-PCR, or by at least two amplicons in MP-PCR. All polymorphisms were determined on the basis of at least two

independent PCR assays with negative controls (primer and water or water alone).

A second *P. nodorum* population also was created for mating-type determination. It originated from leaves collected in Kinston in 2004. Each isolate was derived from a separate leaf, maximizing the probability that all 77 resulting isolates were genetically distinct individuals (22). In all, 11 to 20 isolates were derived from each of five cultivars, in most cases split roughly evenly between two replicates of each cultivar. The cultivars were NC-Neuse (PVP 200400303), P26R12 (PVP 200200234), P26R24 (PVP 200000275), Vigoro Tribute (PI 632689), and USG 3209.

Mating-type determinations were made using primer sequences designed by Bennett et al. (3). The primers produced bands of 550 and 350 bp for MAT-1 and MAT-2, respectively. Mating types were determined for the 26 isolates from 2003 and the 77 isolates from 2004. For 2003, binomial probabilities were calculated to test whether observed proportions of mating types 1 and 2 in the clone-corrected Kinston isolate sample gave reason to doubt an equal distribution of the two mating types. For 2004, χ^2 analysis was performed using SAS PROC FREQ to determine whether mating-type frequencies differed by cultivar.

RESULTS

***P. nodorum* fruiting body frequencies.** From North Carolina samples, 1,031 fruiting bodies were examined from 2003, 1,354 from 2004, and 396 from 2005.

The data for 2003 are presented in Figure 1. In that year, no ascocarps of *P. nodorum* were detected on either basal or upper tissue in the May sample. By July, however, *P. nodorum* ascocarps could be found on both basal and upper tissues. Taking only *P. nodorum* fruiting bodies into account, ascocarps constituted a significantly larger percentage ($\chi^2 = 7.05$, $df = 1$, $P = 0.008$) of fruiting bodies derived from spikes (32.4%) than of those from basal tissue (7.9%). In the August and September samples, ascocarps were again a higher proportion of *P. nodorum* fruiting bodies on spikes than on basal tissues ($P = 0.099$ in August and $P < 0.0001$ in September). In October, only one ascocarp was found in basal tissues; no spikes were sampled.

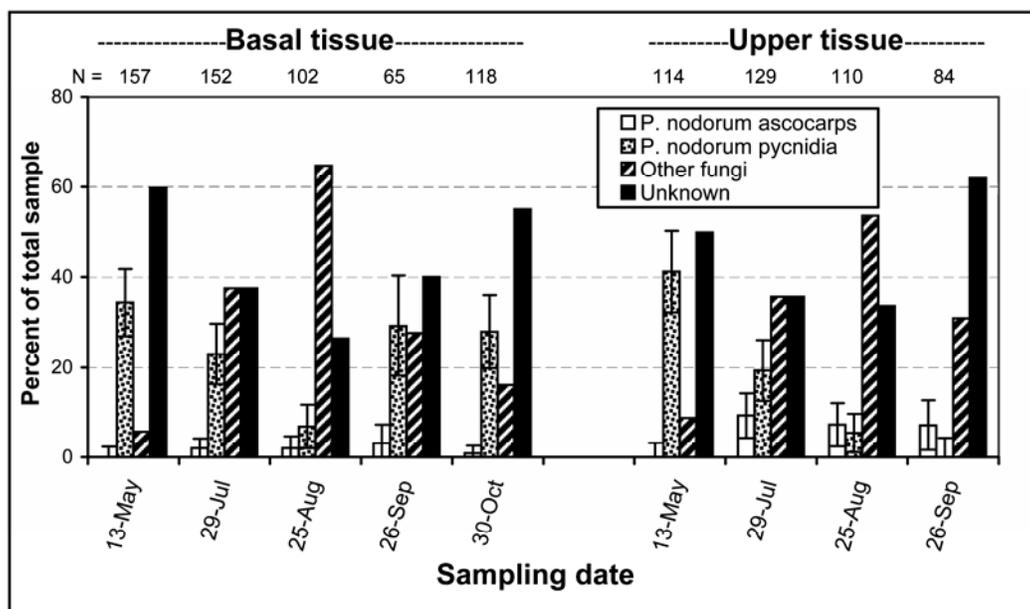


Fig. 1. Frequency of fruiting bodies of *Phaeosphaeria nodorum* and other fungi from basal and upper tissues sampled from winter wheat plants and debris in an experimental field at Kinston, NC, in 2003. N = number of fruiting bodies examined. Error bars show 95% binomial confidence limits on frequencies of *P. nodorum* ascocarps and pycnidia, in each case using the proportion of that fruiting body type in contrast to all other types in the sample. "Unknown" fruiting bodies did not contain spores, and could have been of any species, whereas fruiting bodies classified as "other fungi" contained spores of fungi other than *P. nodorum*.

P. nodorum pycnidia as a percentage of all fruiting bodies in basal tissues dropped to 6.9% in August, but otherwise remained roughly constant throughout the sampling period. In upper tissues, by contrast, *P. nodorum* pycnidia as a percentage of all fruiting bodies dropped from 41.2% in May to 5.5% in August and 0.0% in September.

The percentage of fruiting bodies that contained ascospores or pycnidiospores of fungi other than *P. nodorum* increased from May to July and again in August, then decreased in September and October. Most of these fungi were not identified and were presumably saprophytes. A very small number of fruiting bodies of other wheat pathogens, such as *M. graminicola* (causal agent of Septoria tritici blotch) and *Pyrenophora tritici-repentis* (causal agent of tan spot), were observed.

Small numbers of *Phaeosphaeria nodorum* ascocarps were identified in wheat debris gathered 12 August 2004 and 20 September 2005 in Kinston (Table 1). The relative frequencies of ascocarps in spike and basal tissues were similar to those in the 2003 Kinston samples. No ascocarps were detected in the Salisbury sample collected on 17 June 2004 ($n = 164$) or in the Plymouth sample gathered 15 June 2004 ($n = 424$). One ascocarp was found in the spikes gathered in Salisbury on 11 August 2004 ($n = 115$, 95% BCLs: $0.0\% \leq P \leq 4.8\%$) and none in the basal tissue ($n = 137$).

In the samples from Griffin, GA, *P. nodorum* pycnidia comprised 14 of 100 fruiting bodies examined, or 0.14, from 17 April 2003, and 36 of 179 fruiting bodies, or 0.20, from 2 June 2003 (95% BCLs on proportion of pycnidia versus all other fruiting bodies: $0.08 \leq P \leq 0.23$ and $0.14 \leq P \leq 0.27$, respectively). No *P. nodorum* ascocarps were found (95% BCLs on ascocarps versus all other fruiting bodies: $0.00 \leq P \leq 0.04$ and $0.00 \leq P \leq 0.02$, respectively). Similarly, of the fruiting bodies collected in State College, PA, on 11 July 2003, 33 of 78 fruiting bodies examined, or 0.42, were *P. nodorum* pycnidia, and none were *P. nodorum* ascocarps (95% BCLs: $0.31 \leq P \leq 0.54$ and $0.00 \leq P \leq 0.05$, respectively).

Phaeosphaeria pycnidiospores from all three U.S. states consistently measured 15.4 to 24.2 μm in length and 2 to 2.5 μm in width, and were one- to three-septate. The macroconidia of *P. nodorum* generally measure 15 to 32 by 2 to 4 μm and are zero- to three-septate (12), whereas those of *P. avenaria* f. sp. *triticea* are 26 to 42 by 2.8 to 3.5 μm and three- or, rarely, four-septate (20).

Mating types. After clone-correction, 16 genetically distinct isolates remained from the 26-isolate Kinston sample of 2003. Seven were of MAT-1 and nine were of MAT-2. This ratio of mating types gave no reason to reject the hypothesis of mating-type balance ($P = 0.359$). Of the 77 isolates in the 2004 population, 39 (51%) were of MAT-1 and 38 (49%) of MAT-2. A χ^2 analysis indicated no differences in frequencies of the two mating types by cultivar of origin ($\chi^2 = 4.35$, $df = 4$, $P = 0.36$).

Molecular species identification. After clone-correction, 18 North Carolina isolates were available for species identification. The ITS sequences of all 18 isolates had 98 to 100% similarity with GenBank *P. nodorum* sequences. The sequences of North Carolina isolates that had the greatest divergence from previously published *P. nodorum* ITS sequences (those of STAN11, STAN14, STAN16, STAN17, and STAN24) were deposited in GenBank. Based on sequence similarity, the 18 North Carolina isolates fell into a common group along with other GenBank *P. nodorum* accessions (Fig. 2, group 1). GenBank accessions of *Phaeosphaeria* spp. other than *P. nodorum* consistently grouped separately (Fig. 2, group 2).

DISCUSSION

To our knowledge, this is the first report of *P. nodorum* ascocarps detected in the eastern soft red winter wheat region of the United States. We found low frequencies of the sexual stage in wheat debris throughout the summer following a moderately severe SNB epidemic in North Carolina, and confirmed this finding with more limited samples collected in the following 2 years.

Although our ITS sequence data pertain to a different set of isolates than those originating the fruiting bodies, both samples were derived contemporaneously from the same population. We cannot rule out the presence of *P. avenaria* in North Carolina. However, based on our Kinston sample size of 16, it can be assumed with 95% confidence that *P. avenaria* constitutes less than 17% of the *Phaeosphaeria* population in Kinston. Taken together with conidial dimensions, the sequence data support the conclusion that most, if not all, of the fruiting bodies we identified were of *P. nodorum*, not *P. avenaria* f. sp. *triticea*.

Abundance of precipitation is related to epidemic intensity, and probably to ascocarp frequency (8). During the months of January to July, Kinston received 91.2 cm of rain in 2003, and 84.6% of that amount (77.2 cm) in 2004. In the same months, Salisbury received only 35.4% as much rain in 2004 as in 2003. The dry spring may account for the low frequency of ascocarps detected in the August 2004 sample from Salisbury.

Our data do not indicate the relative importance of sexual reproduction in structuring the *P. nodorum* population in North Carolina. However, for comparison, we can consider the *M. graminicola* population in Oregon, which is essentially random-mating (7). There, ascocarp percentages of *M. graminicola* fruiting bodies derived from basal tissues at the end of three successive growing seasons were 63, 14, and 60%, respectively (8). The epidemics in those 3 years were severe, mild, and severe, respectively. Molecular marker data were used to estimate that 21% of the Oregon *M. graminicola* population were the progeny of sexual reproduction (49,50), a metric different from ascocarp percentage, following a severe epidemic. Using the July 2003 data from the present study, 8% of *P. nodorum* fruiting bodies identified on

TABLE 1. Percentages of *Phaeosphaeria nodorum* and other fruiting bodies identified in basal and spike tissues sampled from winter wheat plants and debris in Kinston, NC, in 2004 and 2005^a

Identified	2004				2005			
	12 August		23 October		16 June		20 September	
	B (%)	S (%)	B (%)	S (%)	B (%)	S (%)	B (%)	S (%)
Fruiting bodies ^b	221	112	108	73	89	84	94	129
<i>P. nodorum</i>								
Ascocarps	0.5 (0.0–2.5)	9.8 (4.3–15.3)	0.0 (0.0–3.3)	0.0 (0.0–4.9)	0.0 (0.0–4.1)	0.0 (0.0–4.3)	0.0 (0.0–3.9)	2.3 (0.5–6.7)
Pycnidia	1.4 (0.3–3.9)	0.0 (0.0–3.2)	4.6 (0.7–8.6)	9.6 (2.8–16.3)	34.8 (24.9–44.7)	86.9 (79.7–94.1)	3.2 (0.0–6.7)	3.9 (1.3–8.8)
Other fungi	20.8	20.5	25.0	5.5	3.4	1.2	30.9	16.3
Unknown	77.4	69.6	70.4	84.9	61.8	11.9	66.0	77.5

^a Basal (B) tissues were lower leaves (F-4 and F-5) and the lower 10 cm of the stems. Spike (S) tissues were glumes, rachis, and peduncles. For reference, harvest dates were 3 June 2004 and 14 June 2005. Numbers in parentheses are binomial upper and lower confidence limits (CLs) on percentage of ascocarps or pycnidia versus all other fruiting bodies. CLs are asymptotic unless the count is 0, when they are exact.

^b Total number of fruiting bodies examined.

basal tissues were ascocarps, and 32% of those on spikes, following a moderately severe epidemic. Thus, sexual recombination may play only a modest role in structuring the North Carolina *P. nodorum* population.

In a facultatively sexual pathogen, the frequency of sexual reproduction may be positively correlated with the degree of host susceptibility (8). It was not possible to ascertain the level of susceptibility of the host genotypes that supplied fruiting bodies for the present study.

P. nodorum ascocarps probably mature in North Carolina wheat crops soon after harvest in June, because they were detected in July to September but not in May or June. During the summers, they may remain dormant in the debris, as evidenced by the roughly constant percentages observed in basal tissues and spikes, respectively, during the July-to-September period. The discharge of *P. nodorum* ascospores is triggered by rainfall (34) if accompanied by moderate temperatures (5 to 25°C) and is reduced at temperatures above 25°C (2). In the wheat-growing areas of North Carolina, mean monthly maximum temperatures are above 25°C from May to September (State Climate Office of North Carolina), and winter wheat emerges in October to December. Spore counts have indicated maximum ascospore release in September to November in Germany (32), August to October in Poland (2), and March to April in France (34). This discrepancy suggests that the role of ascospores in SNB epidemiology may vary according to

environment. Such variation may be substantial within the United States, where there are large north-south differences in the occurrence and timing of temperature extremes, for example.

P. nodorum ascocarps were present at a significantly higher frequency on spikes than on basal tissues. One possible explanation could be a higher infection density (8) on spike tissues which could be caused, in turn, by less competition (*C. Cowger, personal observation*) from powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*) or by differential susceptibility of spike tissues. Spike and leaf susceptibility to *P. nodorum* can be under distinct genetic control (4,15).

The frequency of *P. nodorum* pycnidia declined sharply in August in both basal and upper tissues, and then in September returned to nearly the May level in basal tissues but not in upper tissues. The reason for the disparity between the tissue types is unclear. Other fungi, most of them probably saprophytes, reached similar, maximal percentages in August in both spikes and basal tissues, and then declined in both tissue types in September. Significant frequencies of saprophytic fungi, such as those causing sooty molds, are to be expected on senescing wheat heads (48). Perhaps spikes, which are lying on the ground, disintegrate more rapidly than the still-erect stems, triggering earlier discharge of *P. nodorum* pycnidia.

Why have *P. nodorum* ascocarps not been detected elsewhere in the soft red wheat region? Because *P. nodorum* is heterothallic,

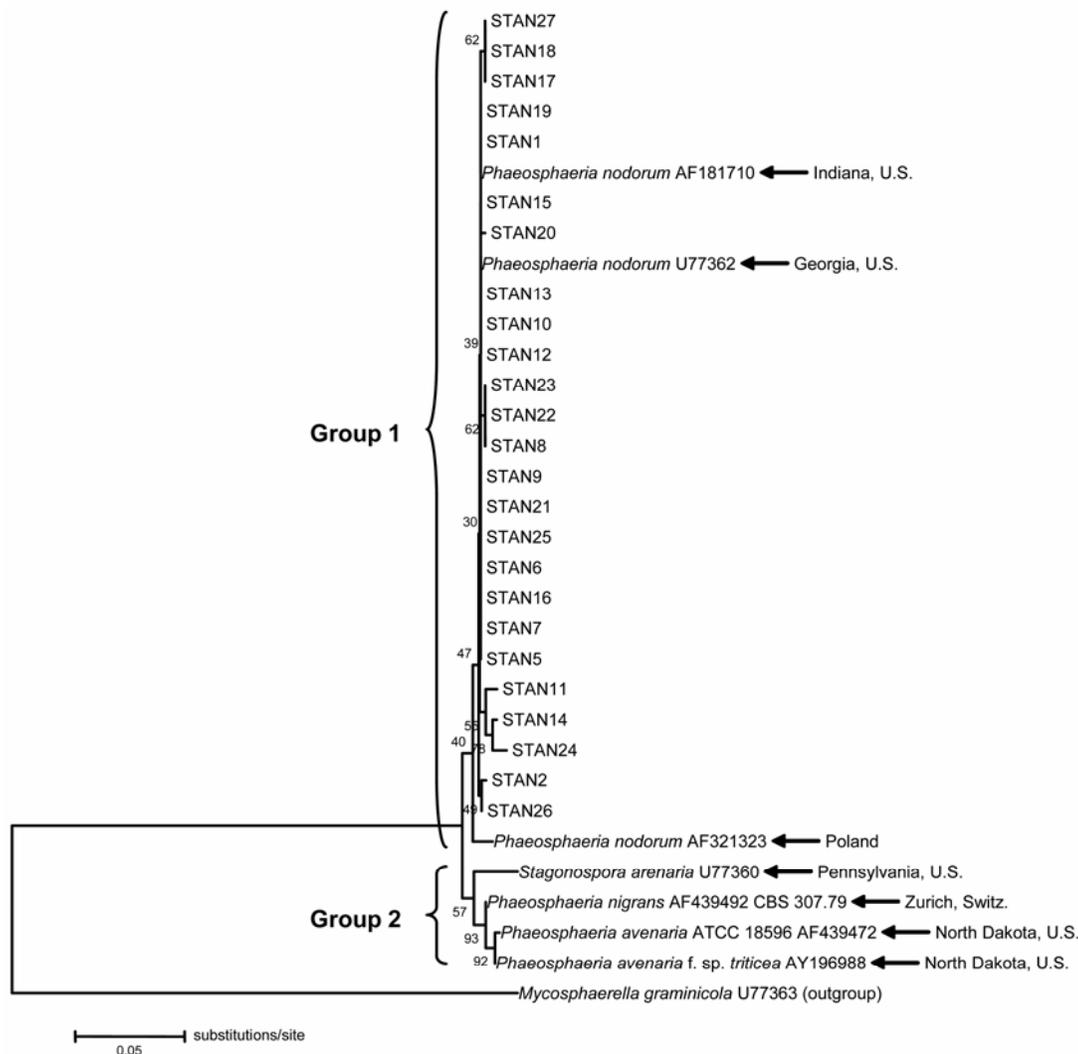


Fig. 2. Phylogenetic tree based on 507 bp of internally transcribed spacer and partial 18S and 28S rRNA sequences of *Phaeosphaeria* isolates, using a *Mycosphaerella graminicola* isolate as the outgroup. STAN isolates 1 to 16 and 22 to 27 were collected in Kinston, NC, and STAN isolates 17 to 21 were collected in Plymouth, NC, in May 2003. Sequences of other isolates were obtained from GenBank (accession numbers given in the text).

the portion of the wheat plant giving rise to ascocarps and the timing with which it does so may be quite specific and dependent on the developmental stage in which adequate infection density occurs. Under some conditions, and in contrast to the present findings, the tissues harboring most ascocarps might be those at the base of plants, which senesce early and are inconspicuous. Indeed, Mehta (31) observed abundant *P. nodorum* pseudothecia mainly on the lower portions of wheat stems and only occasionally on leaves and glumes.

The teleomorph of this pathogen probably requires a relatively long time for maturation, which may severely limit its frequency in some environments. Latent periods have not been reported for *P. nodorum* pseudothecia, but the *M. graminicola*-wheat system provides a relevant comparison. There, ratios of sexual to asexual latent period of between 2 and 3 have been estimated using field observations supported by modeling (11). Asexual latent periods in the *M. graminicola* system have been measured at 17 to 23 days, depending on temperature (40). If these estimates were approximately accurate for *P. nodorum*, a latent period following fertilization (which itself requires the maturation of a previous round of adjacent infections by opposite mating types) of ≈ 2 months would be expected. It is plausible that significant numbers of pseudothecia only can be generated on a given plant part following multiple asexual cycles, which allow infection densities and, thus, probabilities of opposite mating-type encounters to reach adequate levels. This would be particularly true if the inoculum that initiated infections in the late fall or early winter were sparse or imbalanced as to mating type.

Further, interspecies competition may limit the frequency with which *P. nodorum* ascocarps can form in some areas. In the Willamette Valley of Oregon, *P. nodorum* and *M. graminicola* were, until recently, the only important foliar pathogens of winter wheat. In a 3-year survey of *M. graminicola* fruiting bodies collected in Oregon (8), *P. nodorum* pseudothecia composed 5.8, 0.7, and 50.8% of the 2,372 fruiting bodies identified as either *M. graminicola* or *P. nodorum* (C. Cowger, unpublished data). In the eastern United States, the foliar biotrophs *B. graminis* and *Puccinia triticina* often compete with *Phaeosphaeria nodorum* for host tissue. Under those circumstances, *P. nodorum* strains of opposite mating types may encounter each other less frequently, which would limit sexual reproduction.

Another possible explanation for difficulties in finding *P. nodorum* ascocarps in parts of the eastern United States would be an imbalance between the two mating types, although Bennett et al. (3) did not find such an imbalance in New York state. Our data support the hypothesis of mating type balance in North Carolina.

Given the relatively low frequencies of pseudothecia we observed, it is plausible that molecular marker studies would reveal significant departures from random mating in the North Carolina *P. nodorum* population. However, the existence of the teleomorph of *P. nodorum* in North Carolina indicates that, at least in some years, sexual recombination can influence the evolutionary potential of the pathogen.

The presence of a mixed reproductive system with regular sexual and asexual recombination has implications for the durability of resistance (28). Because of higher genotype diversity and the build-up of the fittest clones through polycyclic asexual reproduction, there is a greater likelihood that the pathogen population will adapt to overcome fungicides, resistance genes, and other fungal competitors than if reproduction were strictly asexual.

Resistance breeding programs should focus on quantitative resistance, ideally under polygenic control, because periodic sexual recombination tends to break up co-adapted gene complexes in the pathogen that might be selected for by horizontal host resistance. For artificial inoculation, the most genetically diverse *P. nodorum* population possible should be used, rather than relying on one or two highly pathogenic isolates. Efforts are under-

way to develop an effective artificial field inoculation technique that utilizes a genetically diverse pathogen population.

ACKNOWLEDGMENTS

We thank J. Patton Özkurt for DNA fingerprinting, O. Ibrahim and B. A. McDonald for mating type determinations, G. Bergstrom for advice about *P. avenaria* f. sp. *triticea*, and E. DeWolf and B. Cunfer for leaf collections.

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