

# Genetic Variability in Nuclear DNA in Field Populations of *Stagonospora nodorum*

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## ABSTRACT

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Anonymous nuclear DNA markers were used to characterize the genetic structure of field populations of the wheat pathogen *Stagonospora nodorum*. High levels of genetic and genotypic diversity were found when DNA probes that hybridized to single restriction fragment length polymorphism (RFLP) loci and to a dispersed, repetitive DNA sequence were used. The populations surveyed contained a high number of different genotypes distributed on a small scale. In the majority of cases, separate lesions on the same leaf were caused by different genotypes of *S. nodorum*.

Pairwise comparisons between individual RFLP loci showed that the majority of alleles at these loci were in gametic equilibrium, as is expected in a random-mating population. All of these observations support the hypothesis that ascospores were a significant source of primary inoculum. A probe that hybridizes to a repetitive sequence present on several chromosomes may be useful for differentiating all the genotypes present in a population. These genetic markers will be useful for further studies on the population biology and epidemiology of *S. nodorum*.

*Additional keywords:* gametic disequilibrium, population genetics, wheat glume blotch.

The fungus *Stagonospora nodorum* (Berk.) Castellani & E. G. Germano (= *Septoria nodorum* (Berk.) Berk. in Berk. & Broome, teleomorph *Leptosphaeria nodorum* E. Mueller) causes *Septoria nodorum* glume blotch on wheat (*Triticum aestivum* L.). The disease may cause significant yield losses in years with high rainfall wherever wheat is grown (8). The most comprehensive studies of populations of this pathogen have compared virulence and pathogenicity in collections of isolates originating from different geographic locations. In a study of 33 isolates from eight countries, Scharen et al (20) postulated that geographic populations differed for the frequencies of hypothetical virulence genes on the basis of their responses on a set of 38 wheat differentials. Allingham and Jackson (1) compared the virulence of 282 isolates collected from five counties in Florida on eight wheat cultivars. They found 242 different virulence types among these isolates and a range of different virulence types within each geographic location. However, the amount of genetic variation within a geographic population was difficult to evaluate, because the virulence data were based on the percentage of necrotic leaf area, which is a quantitative rather than a qualitative measure.

Though molecular markers were first used to differentiate isolates of *S. nodorum* more than 25 yr ago (6), it is only in the last 3 yr that they have been used extensively. Cooley and Caten (4) found that electrophoretic karyotypes derived by pulsed-field gel electrophoresis could differentiate five of six *S. nodorum* individuals collected from two adjacent fields. While these data suggested that a high degree of genetic variability may exist within a field, too few isolates were assayed to quantify the amount of genetic variation present in a population. Ueng et al (23) found abundant variation among 11 isolates collected over a 5-yr period using anonymous DNA probes that hybridized to single nuclear restriction fragment length polymorphism (RFLP) loci. However, these 11 isolates represented genetic variation existing in several geographic locations, so they were not indicative of the amount of genetic variation existing within geographic populations.

Our objectives in this experiment were to develop molecular genetic markers suitable for characterizing the population biology of *S. nodorum* and to use these markers to characterize the genetic structure of a population of *S. nodorum*. We describe here the development of single-locus nuclear RFLP markers and moderately repetitive DNA markers that can be used for DNA fingerprinting for *S. nodorum*. These markers were used in combination to analyze genetic diversity in a hierarchical collection of *S. nodorum* isolates sampled from two wheat fields in eastern Texas.

## MATERIALS AND METHODS

**Hierarchical collection of isolates.** Collections of *S. nodorum* were made from two wheat-breeding nurseries in Overton, Texas, in February 1992. In the first field, 31 infected leaves were collected from eight different sites located approximately 10 m apart in the field. Between two and seven leaves were sampled from each site in the first field. At a second field, located approximately 1.5 km distant, three leaves were collected from each of two different sites in the field. Entire leaves were surface sterilized and placed on moistened filter paper in petri dishes at 24 C until cirrhi were exuded from pycnidia. Isolations onto yeast-maltose agar (4 g of yeast extract, 4 g of malt extract, 4 g of sucrose, and 15 g of agar per liter of water) were made from a single lesion on each of 10 leaves. Isolations were made from two or three lesions on each of 27 leaves that had discrete, nonoverlapping lesions. Two isolations were made from different pycnidia within the same lesion for three of the lesions. A total of 68 single-pycnidial isolations were made from 65 lesions in the first field, and 15 isolations were made from 15 lesions in the second field.

**DNA extraction.** Mycelium from each isolate was grown at room temperature for 5-7 days on an orbital shaker in yeast-sucrose broth (10 g of yeast extract and 10 g of sucrose per liter of water). Mycelium was harvested by filtration and lyophilized overnight. DNA was extracted by a modification of the method described by Yelton et al (25). Approximately 1 g of lyophilized mycelium was ground with a mortar and pestle and transferred to a 50-ml centrifuge tube. Ground mycelium was dispersed in

10 ml of extraction buffer (1% SDS [sodium dodecyl sulfate], 100 mM EDTA, 0.1% diethyl pyrocarbonate) and placed in a water bath at 60 C for 60 min. Cell debris was pelleted by centrifuging at 12,000 g for 15 min at 21 C. The supernatant was transferred to a fresh tube and then mixed with 1 ml of 4 M potassium acetate and placed on ice for 45 min. This solution was centrifuged at 28,000 g for 15 min at 4 C, and the supernatant was transferred to a fresh tube. Nucleic acids were precipitated by adding an equal volume of isopropanol and centrifuging at 2,600 g for 10 min at 21 C. The nucleic acid pellet was resuspended in 1 ml of TE (10 mM Tris and 1 mM EDTA) and extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and then once with chloroform-isoamyl alcohol (24:1). The aqueous layer (~500  $\mu$ l) was transferred to a 1.5-ml tube, and nucleic acids were precipitated by adding 2 volumes of 95% ethanol and centrifuging at 12,000 g for 5 min. The final DNA pellet was resuspended in 100–200  $\mu$ l of TE. The average yield was approximately 100  $\mu$ g of DNA from each isolate.

**Development of probes.** A library of random, anonymous DNA fragments was constructed for use as probes. Total DNA from a randomly selected isolate of *S. nodorum* (SN82.19) was digested with *Pst*I, and the fragments were cloned into pUC18 by standard methods (10). Approximately 200 clones were picked randomly to use as probes for RFLP analysis. A preliminary screen of 40 of the clones showed that the pUC18 vector had inserts of *S. nodorum* DNA ranging from 300 to 10,000 bp in size; the average insert size was approximately 3,500 bp. Thirty-four randomly chosen clones were hybridized to *Eco*RI-digested DNA from a collection of 20 isolates of *S. nodorum* sampled from Overton in 1991. On the basis of the results from this preliminary screen (described in Results), eight probes were chosen to characterize the 1992 population. These probes were chosen because they showed strong hybridization to DNA fragments between 500 and 10,000 bp, which represented the optimal range for separation of DNA fragments under our electrophoresis conditions.

**Southern blotting and hybridization.** Five micrograms of DNA from each isolate was incubated overnight with 50 U of *Eco*RI. The DNA fragments were separated on 0.8% agarose gels in TBE (Tris-borate-EDTA) buffer at 2.5 V/cm. Following electrophoresis, the fragments were transferred to Zetaprobe membranes (BioRad, Hercules, CA) by the alkaline transfer method according to the manufacturer's directions.

Membranes were prehybridized for 15 min in a buffer containing 250 mM disodium phosphate and 7% SDS. Probes were labeled with <sup>32</sup>P (Bethesda Research Laboratories, Gaithersburg, MD) by nick translation according to the manufacturer's directions. Probes were hybridized to the membranes overnight in 250 mM disodium phosphate and 7% SDS at 60 C in a hybridization incubator. Following hybridization, membranes were washed twice for 1 h at 60 C in 20 mM disodium phosphate and 5% SDS and then twice for 1 h at 60 C in 20 mM disodium phosphate and 1% SDS.

**Separation of chromosomes by pulsed-field gel electrophoresis.** Protoplasts of *S. nodorum* were prepared as described by Cooley et al (5). Protoplasts were embedded in agarose microbeads by the method of Koob and Szybalski (9) at a concentration of approximately 10<sup>8</sup> protoplasts per milliliter. Chromosomes between 0.5 and 3.5 Mb in size were separated on a BioRad CHEF (contour-clamped homogeneous electric field) DR-II apparatus under the Set B conditions of Morales et al (15), which consisted of a 0.8% agarose gel run in 0.5 $\times$  TBE at 14 C under the following conditions: 92 h, 50 V, 2,700–1,500 s ramp; 26 h, 50 V, 1,500–1,175 s ramp; 28 h, 60 V, 1,175–825 s ramp; 24 h, 80 V, 825–600 s ramp; 24 h, 80 V, 600 s. Chromosome-sized DNA fragments separated by CHEF gels were transferred to nylon membranes and hybridized with *S. nodorum* probes as described above.

**Data analysis.** According to the convention for RFLP analysis, the different restriction fragment size variants detected by each probe were treated as alleles at a single RFLP locus. Each allele was assigned a number according to its frequency in the sample; i.e., the most common allele was assigned number 1, and the

second most common allele was number 2, etc. Loci were analyzed individually and then combined to form a multilocus haplotype, which is a summary of the alleles present at each locus for each isolate (11). DNA fingerprints produced by probe pSNS4 (described in Results) allowed us to identify isolates that were clonal products of asexual reproduction. Only one representative of each clone was used to calculate the clone-corrected allele frequencies for each RFLP locus. Uncorrected allele frequencies were calculated with every isolate in the collection.

Nei's measure of gene diversity (16) was calculated for each locus with the clone-corrected allele frequencies. Genotypic diversity was quantified with Stoddart and Taylor's measure (22):

$$\hat{G} = \frac{1}{n \sum_{x=0} [f_x \cdot (x/n)^2]}$$

where  $n$  is the sample size and  $f_x$  is the number of distinct genotypes observed  $x$  times in the sample. The maximum possible value for  $\hat{G}$ , which occurs when each individual in the sample has a different genotype, is the number of individuals in the sample. To compare  $\hat{G}$  in populations with different sample sizes,  $\hat{G}$  was divided by  $n$  to calculate the percentage of maximum diversity obtained. When genotypic diversity was measured on the basis of DNA fingerprints,  $f_x$  was the number of different DNA fingerprints in the sample. When multilocus haplotypes were used to measure genotypic diversity,  $f_x$  was the number of multilocus haplotypes in the sample.

Gametic disequilibrium, which is a measure of association among pairs of alleles at different loci, can be useful for evaluating the frequency of recombination in populations of haploid organisms that undergo both sexual and asexual reproduction. Disequilibrium between loci was calculated either with the entire collection of isolates or with a single representative of each clone. Measures of gametic disequilibrium were made with methods suggested by Weir (24). Following Weir's notation for the case of multiple alleles, the disequilibrium coefficient ( $D$ ) for alleles  $u$  and  $v$  at different loci was used to compare the observed gametic frequency with the product of the gene frequencies as follows:

$$D_{uv} = p_{uv} - p_u p_v$$

where  $p_{uv}$  was the observed gametic frequency and  $p_u$  and  $p_v$  were the observed frequencies of alleles  $u$  and  $v$  for any two loci. A test for the significance of the disequilibrium coefficient between each pair of alleles at two loci was formulated with the chi-square statistic

$$\chi^2_{uv} = \frac{n \hat{D}_{uv}^2}{\hat{p}_u (1 - \hat{p}_u) \hat{p}_v (1 - \hat{p}_v)}$$

where  $n$  was the number of individuals in the sample and  $\hat{D}_{uv}$  was the maximum likelihood estimator for the coefficient of disequilibrium between alleles  $u$  and  $v$ . The observed allele frequencies for the loci were  $\hat{p}_u$  and  $\hat{p}_v$ , respectively. This chi-square statistic had one degree of freedom. Pairs of alleles that showed a significant ( $P < 0.05$ ) departure from random expectations were tested further with Fisher's exact test of independence (24). The two-tailed exact test for independence between pairs of alleles at different loci was calculated with the computer program FISH6, version 1.001 (7). An association was considered significantly different from zero if the exact test gave a probability less than 0.05. A test for significance of the disequilibrium coefficient across all alleles for each pair of loci was formulated with the chi-square test statistic

$$\chi^2_T = \sum_{u=1}^k \sum_{v=1}^l \frac{n \hat{D}_{uv}^2}{\hat{p}_u \hat{p}_v}$$

for all pairs of alleles *u* and *v*, which were present at loci that had *k* and *l* alleles total, respectively. This chi-square statistic had  $(k - 1)(l - 1)$  degrees of freedom for each pair of loci.

## RESULTS

**Probe screening.** Thirty-two of the 34 probes tested in the preliminary screen of 20 isolates hybridized to one, two, or three DNA fragments in each isolate. Eighteen of these single-locus probes did not detect any polymorphisms. Fourteen of the probes detected from two to five variants at each locus. Seven of these variable probes (Table 1) were selected to analyze the 1992 Overton population. These probes were chosen because 1) they hybridized to DNA fragments 500–11,000 bp in size, which represented the optimal range of resolution under the electrophoresis conditions used; 2) they could resolve all restriction fragments unambiguously; and 3) they each gave a strong hybridization signal.

Two of the probes (pSNS4 and pJSN22) in the preliminary screen hybridized to 10–30 fragments in each isolate. Nearly every isolate in the original screen had a different hybridization pattern with these two probes, suggesting that they might be useful for DNA fingerprinting. The hybridization pattern with the two probes appeared to be identical, suggesting that the same repetitive sequence was cloned twice. Probe pSNS4 was chosen to analyze the 1992 Overton population because it gave a stronger hybridization signal than did pJSN22.

**Genetic diversity in nuclear DNA.** Examples of single-locus and repetitive RFLP data are shown in Figures 1 and 2, respectively. A complete set of single-locus nuclear RFLP data was obtained for only 58 of the 83 isolates as a result of nonspecific background hybridization with some probes and deterioration of the membranes following many cycles of hybridization and

washing. Clone-corrected and uncorrected allele frequencies for each of the seven single RFLP loci are shown in Table 1. The number of alleles per locus ranged from two to four and averaged 3.1 across the seven loci. Nei's measure of gene diversity ranged from 0.27 to 0.66 per locus and averaged 0.45 across the seven RFLP loci. There were 31 different multilocus haplotypes among the 58 isolates that had complete data from all seven loci.

Probe pSNS4 detected 50 different hybridization patterns among the 83 isolates. When we compared the pSNS4 hybridization patterns with the multilocus haplotypes of the 58 isolates that had data for all seven loci, we found that isolates with the same pSNS4 hybridization pattern always had the same multilocus haplotype, i.e., the same alleles at each of the seven individual RFLP loci. However, there were two cases in which isolates with the same multilocus haplotype had different pSNS4 hybridization patterns. This result showed that pSNS4 has a greater potential to discriminate between different genotypes than do multilocus

TABLE 1. Restriction fragment length polymorphism (RFLP) allele designations, approximate fragment sizes, and frequencies in a *Stagonospora nodorum* population from Overton, Texas

| RFLP locus   | Allele | Size (bp) | Uncorrected <sup>a</sup> | Corrected <sup>b</sup> | Nei's measure of gene diversity <sup>c</sup> |
|--|--------|-----------|--------------------------|------------------------|--|
| pJSN35 <i>Eco</i> RI<br><i>n</i> = 75, 44 <sup>d</sup> | 1      | 5,800     | 0.76                     | 0.75                   | 0.375  |
|  | 2      | 7,000     | 0.24                     | 0.25                   |  |
| pJSN121 <i>Eco</i> RI<br><i>n</i> = 75, 47             | 1      | 1,300     | 0.44                     | 0.40                   | 0.658  |
|  | 2      | 6,500     | 0.39                     | 0.36                   |  |
|  | 3      | 5,900     | 0.17                     | 0.23                   |  |
| pJSN27 <i>Eco</i> RI<br><i>n</i> = 81, 48              | 1      | 7,300     | 0.80                     | 0.83                   | 0.293  |
|  | 2      | 6,750     | 0.16                     | 0.13                   |  |
|  | 3      | 3,400     | 0.04                     | 0.04                   |  |
| pJSN3 <i>Eco</i> RI<br><i>n</i> = 82, 50               | 1      | 10,800    | 0.83                     | 0.82                   | 0.306  |
|  | 2      | 9,400     | 0.13                     | 0.14                   |  |
|  | 3      | 8,000     | 0.04                     | 0.04                   |  |
| SNL15 <i>Eco</i> RI<br><i>n</i> = 83, 50               | 1      | 1,750     | 0.35                     | 0.38                   | 0.663  |
|  | 2      | 2,750     | 0.34                     | 0.30                   |  |
|  | 3      | 2,450     | 0.31                     | 0.32                   |  |
| pJSN73 <i>Eco</i> RI<br><i>n</i> = 79, 47              | 1      | 6,400     | 0.86                     | 0.85                   | 0.267  |
|  | 2      | 6,200     | 0.10                     | 0.09                   |  |
|  | 3      | 6,800     | 0.03                     | 0.04                   |  |
|  | 4      | 14,000    | 0.01                     | 0.02                   |  |
| SNL13 <i>Eco</i> RI<br><i>n</i> = 80, 50               | 1      | 2,500     | 0.59                     | 0.54                   | 0.605  |
|  | 2      | 7,700     | 0.28                     | 0.30                   |  |
|  | 3      | 8,200     | 0.10                     | 0.10                   |  |
|  | 4      | 8,800     | 0.04                     | 0.06                   |  |
| Mean   |        |           |                          |                        | 0.452  |

<sup>a</sup>Uncorrected allele frequencies were calculated with all isolates in the sample.

<sup>b</sup>Clone-corrected allele frequencies were calculated with each genotype used only once. DNA fingerprints were used to identify replicates of each genotype.

<sup>c</sup>Calculated for each locus using the clone-corrected sample.

<sup>d</sup>Sample sizes used to calculate uncorrected and clone-corrected allele frequencies, respectively.

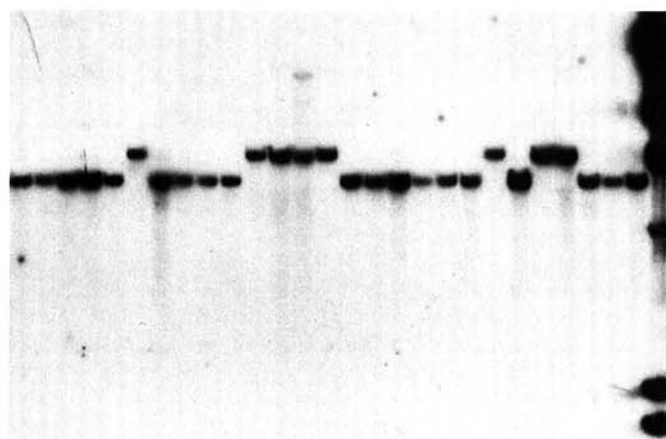


Fig. 1. Autoradiogram showing two alleles at the restriction fragment length polymorphism locus defined by hybridization of probe pJSN35 to DNA digested with *Eco*RI. DNA was from 27 isolates of *Stagonospora nodorum* sampled from a single field. The last lane is a lambda size standard.

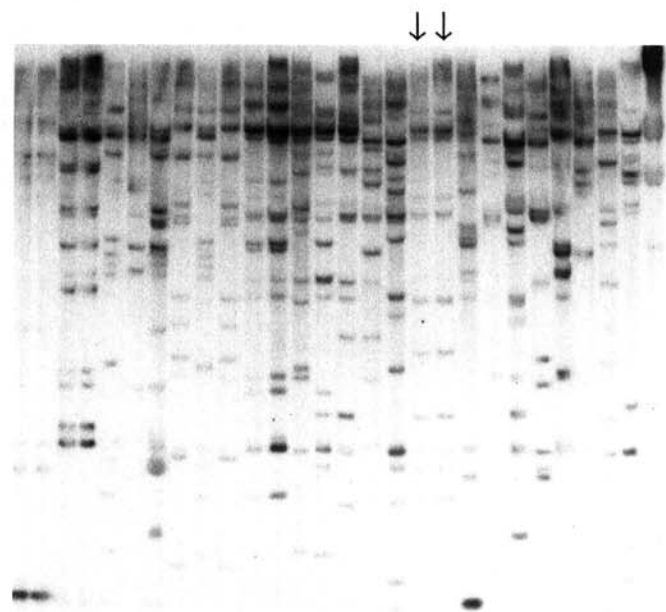


Fig. 2. Autoradiogram showing DNA fingerprints generated by hybridization of probe pSNS4 to DNA digested with *Eco*RI. DNA was from the same 27 isolates of *Stagonospora nodorum* shown in Figure 1. Arrows indicate an example of isolates having the same DNA fingerprint. These isolates, which also have the same alleles at each of seven single restriction fragment length polymorphism loci, are probably clones.



haplotypes on the basis of seven individual loci. Because pSNS4 hybridizes to many polymorphic loci simultaneously and it appears to differentiate most or all of the genotypes in a population, we refer to these complex hybridization patterns as "DNA fingerprints" throughout the remainder of the paper.

The CHEF electrophoresis parameters resolved nine of the 15-19 chromosomes of *S. nodorum* (Fig. 3A). Probe pSNS4 hybridized to at least five chromosomes, demonstrating that the multigene family that has sequence homology with pSNS4 is dispersed among several different chromosomes in the nuclear genome (Fig. 3B).

The frequency distributions of isolates having the same multilocus haplotypes or DNA fingerprints are shown in Table 2. These distributions were used to calculate two measures of genotypic diversity in the population. From the haplotype data, we calculated  $\hat{G} = 20$ , which was 34% of the maximum possible value of 58. From the DNA fingerprint data, we calculated  $\hat{G} = 35$ , which was 42% of the maximum possible value of 83.

A summary of the measures of gametic disequilibrium is shown in Table 3. The number of pairs of loci having significant non-random associations among alleles was larger when all isolates were included in the data set than when only a single representative of each clone was used in the analysis. In general, both types of analyses indicated a low level of disequilibrium among these RFLP loci. For the data set that included multiple representatives of each clone, 37 of 206 (18%) tests between pairs of alleles were significant at the 5% level with Weir's  $\chi^2_{uv}$ , but only 13 of these 37 were significant at the 5% level with Fisher's exact test. Nine of 21 (43%) tests between pairs of loci ( $\chi^2_{\bar{r}}$ ) were significant at the 5% level. For the data set that included a single representative of each clone, 15 of 206 (7%) tests between pairs of alleles were significant at the 5% level with Weir's test, but only 3 of these 15 were significant at the 5% level with Fisher's exact test. Three of 21 (14%) tests between pairs of loci were significant at the 5% level.

## DISCUSSION

This experiment showed that field populations of *S. nodorum* may contain a high level of genetic variation distributed on a small scale. Fifty different nuclear DNA genotypes were detected on 37 different leaves collected from two fields. Nei's measure of gene diversity indicated that the level of diversity present at individual RFLP loci was high. A similar high level of diversity was found at anonymous RFLP loci in California and Oregon populations of the wheat pathogen *Mycosphaerella graminicola* (3,11). Stoddart's measure of genotypic diversity was 34% of its maximum value on the basis of multilocus haplotypes. For comparison, a similar multilocus analysis of RFLP loci in a population of *M. graminicola* isolates originating from a field in Oregon

showed that Stoddart's measure was 62% of its maximum value (3). The difference in genotypic diversity between these populations is probably due mainly to differences in sampling and in the number of individual RFLP loci tested. In the Oregon population, a single isolation was made from each leaf, 11 RFLP

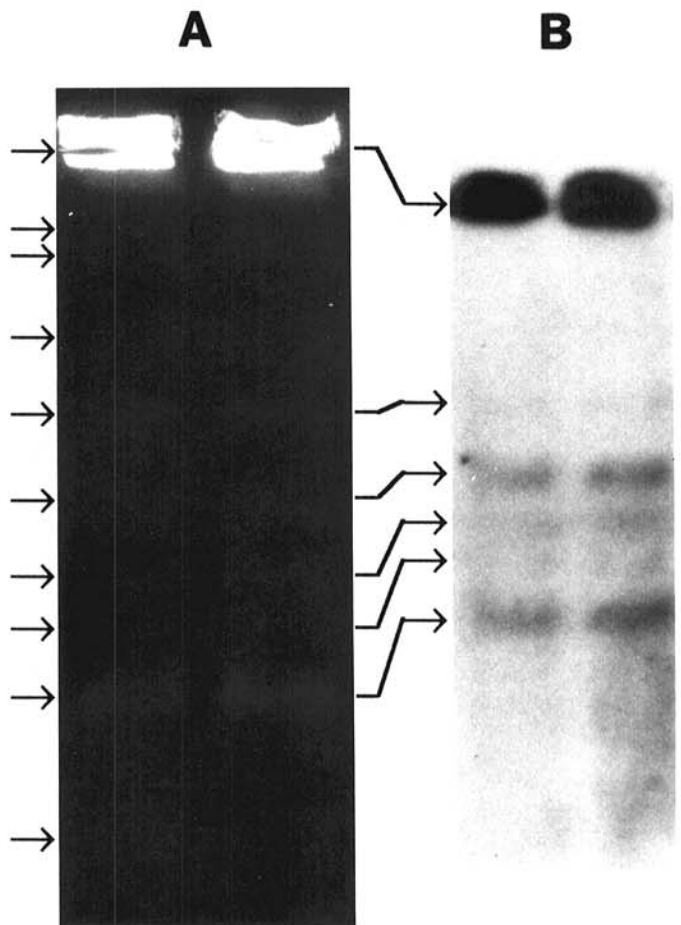


Fig. 3. Physical evidence that pSNS4 hybridizes to a multigene family distributed across several electric chromosomes. Panel A, CHEF (contour-clamped homogeneous electric field) gel showing chromosomes of *Stagonospora nodorum* isolate XA1.1 separated according to the Set B conditions of Morales et al (15). Both lanes contain DNA from isolate XA1.1. The top arrow indicates the origin of the gel. Other arrows indicate positions of chromosome-sized DNA molecules. Panel B, Southern blot of CHEF gel shown in Panel A that was hybridized with pSNS4. Arrows indicate chromosomes that show a hybridization signal.

TABLE 2. Frequency distribution of genotypes in an Overton, Texas, population of *Stagonospora nodorum*<sup>a</sup>

| $x$                                | $f_x$<br>(haplotypes) <sup>b</sup> | $(f_x) \cdot (x/n)^2$<br>(haplotypes) | $f_x$<br>(fingerprints) <sup>c</sup> | $(f_x) \cdot (x/n)^2$<br>(fingerprints) |
|------------------------------------|------------------------------------|---------------------------------------|--------------------------------------|---|
| 1                                  | 16                                 | 0.005                                 | 30                                   | 0.004                                   |
| 2                                  | 10                                 | 0.012                                 | 14                                   | 0.008                                   |
| 3                                  | 3                                  | 0.008                                 | 2                                    | 0.003                                   |
| 4                                  | 0                                  | 0                                     | 2                                    | 0.005                                   |
| 5                                  | 1                                  | 0.007                                 | 1                                    | 0.004                                   |
| 6                                  | 0                                  | 0                                     | 1                                    | 0.005                                   |
| 8                                  | 1                                  | 0.019                                 | 0                                    | 0                                       |
| Total number of genotypes          | 31                                 | ...                                   | 50                                   | ...                                     |
| Total number in sample             | 58                                 | ...                                   | 83                                   | ...                                     |
| $\sum_{x=0}^n [f_x \cdot (x/n)^2]$ | ...                                | 0.051                                 | ...                                  | 0.029                                   |
| $\hat{G}$                          | 20                                 | ...                                   | 35                                   | ...                                     |

<sup>a</sup> An index of genotypic diversity ( $\hat{G}$ ) based on the number of times ( $x$ ) each genotype is present and the number of different genotypes ( $f_x$ ) present in a sample of size  $n$  (21).

<sup>b</sup> Multilocus haplotypes were differentiated by combining the alleles present at seven individual restriction fragment length polymorphism loci.

<sup>c</sup> DNA fingerprints were based on hybridization of probe pSNS4 to DNA from each isolate.

loci were assayed, and 315 different multilocus haplotypes were found in a sample of 406 isolates. In the Overton population, several isolations were made from most leaves, seven RFLP loci were assayed, and 31 haplotypes were found in a sample of 58 isolates. Both populations contained a high degree of genotypic diversity.

Though sample sizes from individual field locations were too small to conduct a meaningful hierarchical analysis of gene diversity, the distribution of genotypes in the field was consistent with the hypothesis that the majority of genetic variation was distributed on a small scale. In the field location with the largest sample size, 11 different genotypes were found among 15 isolates. On average, 5.5 different genotypes were present in a sample of 8.3 isolates collected from the 10 locations in the two fields. In 70% of the tests for variation among lesions on a single leaf, we found different genotypes present on the same leaf. In the three tests for variation within a lesion, the two isolates taken from the same lesion had the same DNA fingerprint. While the DNA fingerprint data showed that asexual reproduction occurred in field populations of *S. nodorum*, the most common clone made up only 7% of the sample. All of these data suggest that no genotype occurs at a high frequency in a field population of *S. nodorum*. Rather, the pathogen population appears to be arranged as a mosaic of overlapping clusters of clones, as described previously for *M. graminicola* (11).

The amount and distribution of genetic variation in field populations of *S. nodorum* were strikingly similar to those found in the small grain pathogens *M. graminicola* (11) and *Rhynchosporium secalis* (14). All three of these fungi produce asexual spores that are dispersed by rain splash, and each is a generalist pathogen that can infect several different grass species. The ascospores of *S. nodorum* and *M. graminicola* are well suited for wind dispersal, but *R. secalis* is not known to have a sexual stage in its life cycle. For each of these three pathogenic fungi, hierarchical sampling was combined with RFLP and/or isozyme genetic markers to determine the distribution of genetic variation in cultivated fields. For each pathogen, we found that 1) field populations contained a high number of different genotypes; 2) genetic variation was distributed on a small scale within a field with many different genotypes present on the same leaf and sometimes in the same lesion; and 3) clusters of genetically identical individuals were found grouped in the same location in the field.

For *M. graminicola*, it was hypothesized that these data were consistent with a disease cycle in which the air-dispersed asco-

spores were the main primary inoculum source and the splash-dispersed pycnidiospores were the secondary inoculum source of an epidemic (11). We now consider the possibility that ascospores are the main source of primary inoculum and pycnidiospores are the secondary inoculum source for *S. nodorum*. The distribution of clones agrees with previous epidemiology studies that suggested wind as the means of dispersal of the primary inoculum (19,21). Though this experiment was not designed to determine the relative importance of sexual and asexual inoculum, the finding of so many different multilocus genotypes, and few widely distributed clones, is consistent with the hypothesis that sexual ascospores are a significant source of inoculum in the field.

The gametic disequilibrium analysis also supports the hypothesis that these individuals originated from a random-mating population. For diploid organisms, deviations from Hardy-Weinberg equilibrium can be used to indicate nonrandom mating. Tests for Hardy-Weinberg equilibrium measure departures from expectation for the number of heterozygous individuals in a population. Haploid organisms such as *S. nodorum* do not exhibit heterozygosity, but measures of associations between alleles at different loci (i.e., measures of gametic disequilibrium) offer similar tests for departures from random mating. A population that reproduces mainly by asexual reproduction will be composed of a limited series of clonal lineages. Repeated sampling of the same clone in a population will generate disequilibrium, because all of the alleles in that clone will be linked. A population that reproduces mainly by sexual reproduction undergoes regular meiosis that independently assort alleles at unlinked loci each sexual generation. This leads to random associations among alleles at different loci.

In our analysis, disequilibrium due to sampling the same clone is reflected in the finding that 18% of the 206 comparisons among alleles at different RFLP loci had nonrandom associations when the entire collection of isolates (in which many clones were present more than once) was used to measure gametic disequilibrium (Table 3). Only 6% of the 206 associations were significant when the more rigorous Fisher's exact test was used. When the effects of sampling the same clone were removed by using the clone-corrected sample, only 7% of the 206 comparisons showed significant associations with the chi-square test, and none of these was significantly associated when the exact test was used (Table 3). The conclusion from both of these analyses is that the great majority of alleles at these loci are randomly associated (i.e., in gametic equilibrium), which supports that hypothesis that these

TABLE 3. Measures of gametic disequilibrium among pairs of restriction fragment length polymorphism (RFLP) loci in an Overton, Texas, population of *Stagonospora nodorum*<sup>a</sup>

| Probe  | Probe                  |   |                       |                        |                         |                        |                       |
|--------|------------------------|---|-----------------------|------------------------|-------------------------|------------------------|-----------------------|
|        | JSN35                  | JSN121                                    | JSN27                 | JSN3                   | SNL15                   | JSN73                  | SNL13                 |
| JSN35  |                        | 0/6 <sup>b</sup><br>0.70 (2) <sup>c</sup> | 2/6 (0)<br>9.57** (2) | 2/6 (0)<br>9.13** (2)  | 0/6<br>0.41 (2)         | 0/8<br>2.08 (3)        | 2/8 (0)<br>5.48 (3)   |
| JSN121 | 0/6<br>0.43 (2)        |   | 0/9<br>6.74 (4)       | 0/9<br>2.78 (4)        | 0/9<br>6.27 (4)         | 3/12 (0)<br>15.1* (6)  | 0/12<br>0.71 (6)      |
| JSN27  | 3/6 (1)<br>12.67** (2) | 2/9 (0)<br>11.23* (4)                     |                       | 0/9<br>0.85 (4)        | 0/9<br>3.26 (4)         | 1/12 (0)<br>1.76 (6)   | 2/12 (0)<br>7.57 (6)  |
| JSN3   | 3/6 (0)<br>10.29** (2) | 0/9<br>5.08 (4)                           | 0/9<br>1.74 (4)       |                        | 1/9 (0)<br>7.64 (4)     | 0/12<br>3.60 (6)       | 1/12 (0)<br>6.31 (6)  |
| SNL15  | 2/6 (0)<br>2.86 (2)    | 4/9 (4)<br>17.57** (4)                    | 2/9 (1)<br>10.20* (4) | 3/9 (1)<br>14.52** (4) |                         | 0/12<br>3.59 (6)       | 0/12<br>7.49 (6)      |
| JSN73  | 0/8<br>2.98 (3)        | 2/12 (0)<br>16.47* (6)                    | 0/12<br>2.67 (6)      | 1/12 (0)<br>3.46 (6)   | 0/12<br>5.90 (6)        |                        | 2/16 (0)<br>14.26 (9) |
| SNL13  | 0/8<br>5.16 (3)        | 0/12<br>4.45 (6)                          | 3/12 (0)<br>12.49 (6) | 1/12 (0)<br>8.24 (6)   | 5/12 (4)<br>17.93** (6) | 5/16 (2)<br>22.58* (9) |                       |

<sup>a</sup>Numbers above the diagonal are measures of disequilibrium calculated with only one representative of each clone. Numbers below the diagonal are measures of disequilibrium calculated with all isolates.

<sup>b</sup>The first row shows the number of significant ( $P < 0.05$ ) chi-square tests between individual alleles at different RFLP loci over the total number of tests made. The number in parentheses is the number of tests that were also significant with Fisher's exact test.

<sup>c</sup>The second row shows the result of a chi-square test for the significance of association between all alleles at the two loci. The number in parentheses is the degrees of freedom for this test. \* = Significant at  $P < 0.05$ ; \*\* = significant at  $P < 0.01$ .

isolates originated from a random-mating population.

The genetic structure of these populations may have important implications for plant breeding and fungicide screening programs. If a large amount of genetic variation is distributed on a small scale, then these pathogen populations probably contain sufficient genetic variation to evolve rapidly in response to a changing environment. Under this scenario, a cultivar containing a single, pathotype-specific resistance gene is not likely to last long when grown in a monoculture. Similarly, resistance to fungicides may evolve rapidly in these populations. It is obvious from these results that screening programs should utilize a large, genetically diverse group of strains when resistance genes or fungicides are being tested in field or greenhouse experiments.

This experiment also may provide some insight into the epidemiology of *S. nodorum*. The most common genotype identified by DNA fingerprints was sampled five times from two different sites in the first field and one time in the second field. Four other genotypes were found at two different sites in the first field. Finding the same genotype at different sites in the same field suggests that the asexual pycnidiospores have the potential to move at least 10 m (the distance between adjacent sampling sites) over the course of the growing season. We consider it less likely that splash dispersal could move spores between fields separated by 1,500 m. It is more likely that pycnidiospores were moved between these fields on contaminated machinery or clothing during routine maintenance of experimental plots. It is also possible that seed infected by the same genotype was planted in the two different fields, because the same seed source was used to plant both fields.

The RFLP markers described in this paper will provide useful tools for studying the population genetics of *S. nodorum* and for monitoring particular genotypes in field or greenhouse studies. A previous study describing the development of nuclear RFLP markers in *S. nodorum* found that 39% of 56 random probes detected polymorphisms among 11 isolates from different geographic locations (23). We found a similar proportion of polymorphic probes; 47% of the 34 probes detected polymorphisms among 20 isolates originating from the same field. Two of the 34 probes, which hybridized to the same dispersed family of repetitive sequences, appear to be useful for DNA fingerprinting. The distribution of these sequences among several different chromosomes was similar to the distribution of repetitive sequences used for DNA fingerprinting in *M. graminicola* (13). Though we found repetitive elements less frequently than did McDonald and Martinez in their study of *M. graminicola* (12), our finding of a repetitive DNA family after testing only 34 probes suggests that other repetitive elements may be present in the *S. nodorum* genome.

These genetic markers may be especially useful for testing hypotheses about the effect of host genotype on pathotype evolution in *S. nodorum*. Previous studies with *S. nodorum* suggested that passing through a wheat or barley host led to a change in virulence between wheat-adapted and barley-adapted isolates (18). The RFLP markers used in our study may be useful for tagging a high number of selectively neutral loci in these isolates to determine whether changes in virulence were a result of contamination (as proposed by Osbourn et al [17]) or of selection (as proposed by Berecquet et al [2]). Similarly, these markers may be used in future experiments to monitor the movement of individual genotypes and different pathotypes in replicated field experiments. We anticipate using these markers to study gene flow among populations of *S. nodorum* in North America, as we described in our report on experiments with *M. graminicola* (3).

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