Correlation Between Molecular and Biological Characters in Identifying the Wheat and Barley Biotypes of *Stagonospora nodorum*

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ABSTRACT

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Thirty-five isolates of *Stagonospora nodorum* from barley, wheat, and other hosts were identified as either the barley or wheat biotypes based on cultural characters and host specificity. Identification of the biotypes based on these phenotypic criteria was compared with genetic identification by analysis of restriction fragment length polymorphisms (RFLPs). Twenty-six probes randomly isolated from genomic DNA of a barley biotype isolate were used in the hybridization to differentiate the barley and wheat biotypes. Eighteen and 17 of the 26 probes detected no variation among 14 isolates of the barley biotype and 13 isolates of the wheat biotype, respectively, and unambiguously differentiated the two biotypes. Genetic distance, an estimate of accumulated number of allele substitutions per locus, is 2.286 between the barley and wheat biotypes. The genetic similarity was very high (>0.82) within each of the two biotypes and was very low (0.12) between the two biotypes. Clustering of the isolates based on individual hybridizing DNA bands correlated with biotype identification based on biological characters. The wheat and barley biotypes were then compared with isolates of *Stagonospora avenae* f. sp. *triticea* and an isolate of *Stagonospora arenaria* by hybridizing with 31 anonymous DNA probes (11 from a wheat biotype, 12 from a barley biotype, and eight from *S. a. triticea*). Two isolates of *S. nodorum* of unknown biotype, SN91-X and SNO38NY-89, used in a previous study, were considered to be the wheat biotype based on DNA banding patterns. Based on allele frequencies of the 31 RFLP loci, the estimated genetic distance between the barley biotype and the wheat biotype of *S. nodorum* was as great as between any of the two biotypes and *S. a. triticea*. Likewise, analysis based on discrete hybridizing bands showed that the barley biotype had a closer relationship with *S. a. triticea* than with the wheat biotype. Two probes from the barley biotype were specific for the barley biotype and did not detect any DNA bands in the wheat biotype. These barley biotype-specific probes may be used as natural markers for identification of the barley biotype.

Additional keywords: gene identity, *Leptosphaeria nodorum, Phaeosphaeria nodorum, Septoria nodorum.*

Stagonospora nodorum (Berk.) Castellani & E.G. Germano (teleomorph *Phaeosphaeria nodorum* (E. Müller) Hedjaroude) causes a leaf and glume blotch on wheat (*Triticum aestivum* L.) (7,37,45), barley (*Hordeum vulgare* L.) (9,15,17–20,30,35,46), and other small grains (37,46). Wheat- and barley-adapted biotypes of the fungus have been differentiated on the basis of several characteristics. The two biotypes are identical in conidial size and shape (9,15,35) but differ in pathogenicity (9,15,32,35). The barley biotype typically is pathogenic to barley and nonpathogenic to wheat and triticale (9). The wheat biotype is pathogenic to wheat and triticale. Isolates of the wheat biotype range from nonpathogenic (15,35,45,46) to moderately pathogenic to barley, and this biotype is often isolated from barley leaves and seed (8,9,28). Isolates pathogenic to wheat or barley were proposed to be two forma speciales of *S. nodorum* (35). However, it has been reported that the pathogenicity of one biotype toward the alternative host increased after passage through the alternative host several times by artificial inoculation and reisolation (13,32). Isolates of the two biotypes differ in colony characters, isozyme migration rate by gel electrophoresis for hexokinase and alkaline phosphatase, growth rate at 31 C, and fluorescence under near-ultraviolet light (9,26,28). Intermediate isolates with one or more characters typical of each biotype are occasionally found (28).

Biological aspects of the barley biotype of *S. nodorum* have been determined during the past 24 yr, but this biotype's genetic relationship to the wheat biotype and related *Stagonospora* species is still unclear. The objectives of this study were 1) to determine if isolates of *S. nodorum* from barley and other hosts characterized as either the barley biotype or the wheat biotype by host range and cultural characters can be differentiated by restriction fragment length polymorphism (RFLP) analysis, 2) to evaluate the genetic variability among isolates of the barley biotype recovered over 13 yr from several locations in the southeastern United States, and 3) to infer the relationship of the two biotypes of *S. nodorum* with *Stagonospora avenae* f. sp. *triticea,* another important cereal leaf pathogen. Preliminary results have been reported (43,44).

MATERIALS AND METHODS

Fungal isolates and maintenance. Twenty-five isolates of *S. nodorum* were collected from barley at 16 locations in five southeastern states (Table 1). Seven isolates of *S. nodorum* from wheat, two from triticale (× *Triticosecale* Wittmack), two from little barley (*Hordeum pusillum* Nutt.), and one from ryegrass (*Lolium* sp.) were also collected. Six isolates of *S. a. triticea* from wheat, two from foxtail barley (*Hordeum jubatum* L.), three from rye (*Secale cereale* L.), and one isolate of *Stagonospora arenaria,* the cause of purple leaf spot in orchardgrass (*Dactylis glomerata* L.) (47), were also tested. Isolates, except those from American

Type Culture Collection (Rockville, MD), were purified by the single-spore technique and stored by lyophilization.

Determination of host range and cultural characteristics. Thirty-five isolates of *S. nodorum* (all except SN91-X and SNO38NY-89 from Table 1) were characterized for host range by inoculation on detached leaves of wheat, barley, rye, oat, and triticale using the procedure of Benedikz et al (2). The cultivars used in pathogenicity tests were Holley wheat, Milton barley, Wrens Abruzzi rye, Mitchell oat, and Sunland triticale. Each isolate was grown on Czapek-Dox V8 agar following standard methods (9). Two sites per 60 mm leaf segment of each suscept were inoculated by placing 3-μl droplets containing 10^6 conidia per milliliter on the surface. Two leaf segments of each suscept were inoculated for a total of four inoculation sites. Lesion length was measured 9–12 days after inoculation. The pathogenicity tests were conducted twice for each suscept–isolate combination. Isolates from ryegrass and little barley were also inoculated on those respective hosts in preliminary tests to confirm their pathogenicity. Culture morphology and fluorescence were determined on Czapek-Dox V8 agar and oxgall agar, respectively, as previously described (9).

DNA isolation and genomic library construction. The fungal cultures were first grown on Czapek-Dox V8 agar supplemented with 5% complete supplement (27) at 21 C with constant near-ultraviolet illumination. A mycelial agar block was transferred to a yeast-malt-sucrose liquid medium (0.5% yeast extract, 0.5% malt extract, and 2% sucrose) and incubated with constant shaking (120 rpm) for 7–10 days at room temperature. Mycelia were harvested by filtration and centrifugation, and total genomic DNA was isolated using procedures described earlier (41,42). A DNA

TABLE 1. Isolates of *Stagonospora* species used in restriction fragment length polymorphism (RFLP) analysis

Species Isolate	Geographic origin	Year of isolation	Host plant	Biotype[a]	DNA type[a]
S. nodorum					
SN91-X[b]	Tippecanoe Co., IN	1991	Wheat	nd	W
SN038NY-89[b]	Orleans Co., NY	1989	Wheat	nd	W
S-74-20A	Griffin, GA	1975	Wheat	W	W
S-79-4	Tifton, GA	1979	Triticale	W	W
S-80-301	Williamson, GA	1980	Triticale	W	W
S-80-607	Williamson, GA	1980	Little barley	W	W
S-81-B10B[c]	Houston Co., GA	1981	Barley	W	W
S-81-W12[b]	Marion Co., OR	1981	Wheat	W	W
S-81-B13B	Griffin, GA	1981	Barley	W	W
S-82-15B[c]	Griffin, GA	1982	Little barley	W	W
S-82-18B	Huntsville, AL	1982	Ryegrass	W	W
S-82-12	Huntsville, AL	1982	Wheat	W	W
S-83-5[c]	Athens, GA	1983	Barley	W	W
S-86-1	Tamaqua, PA	1986	Wheat	W	W
S-89-3	Griffin, GA	1989	Wheat	W	W
S-92-10[c]	Raleigh, NC	1992	Barley	W	W
S-92-2	Fayetteville, AR	1992	Barley	W	W
S-92-9[c]	Raleigh, NC	1992	Barley	W	W
S-92-11	Raleigh, NC	1992	Barley	W	W
S-92-8	College Park, MD	1992	Barley	W	W
S-80-603	Williamson, GA	1980	Barley	B	B
S-80-611[c]	Laurinburg, NC	1980	Barley	B	B
S-81-B9	Clayton, NC	1981	Barley	B	B
S-82-13[c]	Senoia, GA	1982	Barley	B	B
S-83-2[bc]	Tifton, GA	1983	Barley	B	B
S-83-3	Plains, GA	1983	Barley	B	B
S-83-6	Athens, GA	1983	Barley	B	B
S-83-7[c]	Holland, VA	1983	Barley	B	B
S-83-8	Holland, VA	1983	Barley	B	B
S-83-9	Warsaw, VA	1983	Barley	B	B
S-84-2	Moultrie, GA	1984	Barley	I	B
S-84-3	Moultrie, GA	1984	Barley	B	B
S-92-4	College Park, MD	1992	Barley	B	B
S-92-5	Raleigh, NC	1992	Barley	B	B
S-92-7[c]	Raleigh, NC	1992	Barley	B	B
S-93-38[c]	Calhoun, GA	1993	Barley	B	B
S-93-39	Williamson, GA	1993	Barley	B	B
S. avenae f. sp. *triticea*					
SAT002NY-84[a]	New York	1984	Wheat	na	na
S-81-W10[b]	Washington	1969	Wheat	na	na
S-81-510	Benson, ND	1981	Wheat	na	na
ATCC18596[b]	North Dakota	Unknown	Wheat	na	na
ATCC26371[b]	Minnesota	Unknown	Wheat	na	na
ATCC26372	Minnesota	Unknown	Wheat	na	na
ATCC26374	Minnesota	Unknown	Foxtail barley	na	na
ATCC26377	Minnesota	Unknown	Foxtail barley	na	na
ATCC26378[b]	Minnesota	Unknown	Rye	na	na
ATCC26380	Minnesota	Unknown	Rye	na	na
ATCC26383	Minnesota	Unknown	Rye	na	na
S. arenaria					
1444	Centre Co., PA	1980	Orchardgrass	na	na

[a] Biotype was determined based on host range, colony type, and fluorescence (see Table 2). DNA type was based on cluster analyses (Figs. 1 and 2). W = wheat biotype, B = barley biotype, I = intermediate biotype, nd = not determined, and na = not applicable.

[b] Isolates used in a previous study (42).

[c] Isolates not included in the hybridization study in comparison with other *Stagonospora* species.

library was constructed from barley biotype isolate S-83-2 (Table 1). *Eco*RI enzyme restriction, DNA ligation, bacterial transformation, selection, and purification of cloned plasmid DNA were performed as described previously (41).

DNA hybridization. DNA hybridization experiments were divided into two independent studies: differentiation between the wheat and barley biotypes (study 1), and genetic relatedness among the three *Stagonospora* species (study 2). For study 1, 26 anonymous DNA probes were randomly selected from a DNA library developed from isolate S-83-2. The probes were used to hybridize with 14 isolates of the barley biotype and 13 isolates of the wheat biotype. In study 2, 38 isolates representing the two biotypes of *S. nodorum, S. a. triticea,* and *S. arenaria* were included in hybridization with 31 DNA probes. Of the 31 probes, 11 were from the wheat biotype (42), 12 from the barley biotype, and eight from *S. a. triticea* (42). All probes, except SNBE39, hybridized to one or two DNA bands in each isolate.

Total DNA enzyme digestion, agarose gel electrophoresis, and gel blotting onto membranes were performed as described previously (36,41). *Eco*RI-digested plasmid DNA (0.5 mg each) containing the probe was ^{32}P-labeled using the random-priming technique (10,11). Membrane hybridization, washing conditions, and film exposure were as reported previously (41).

Data analysis. Two approaches were taken to analyze the data generated in DNA hybridization studies. The first approach is based on allelic frequencies. Following the convention of RFLP analysis, the various DNA banding patterns detected by each anonymous probe were considered to be alleles of that putative RFLP locus. The allele frequencies for each population were calculated. Three populations were compared with themselves: the barley biotype and the wheat biotype of *S. nodorum* and the isolates of *S. a. triticea.* The single isolate of *S. arenaria* was not compared in this approach. The proportion of polymorphic loci is given by the formula $P = x/r$ where x is the number of polymorphic loci and r is the total number of loci examined (22). Nei's measure of gene identity (24) of each locus was calculated for each population. Gene identity (J) is defined as the probability that two randomly chosen alleles from a population will be identical, and is given by the formula $J = \Sigma p_j^2$ where p_j is the frequency of the jth allele. The arithmetic mean (J_m) of J's of all loci (including monomorphic loci) and allele frequencies were used to compare populations in calculating the normalized identity (I), which estimates the proportion of genes that are identical in structure in two populations ("identical" here means "indistinguishable," not "identical by descent"). The quantity of I is given by the formula:

$$I = \frac{(\Sigma p_j q_j)/r}{\sqrt{J_{m1} J_{m2}}}$$

where p_j and q_j are frequencies of the jth allele in populations 1 and 2, respectively; r is the number of loci examined; and J_{m1} and J_{m2} are the mean gene identity of populations 1 and 2, respectively. Genetic distance, D, between the populations can be measured by $D = -lnI$, which estimates the number of allelic

TABLE 2. Biotype characterization of isolates of *Stagonospora nodorum* based on colony morphology and host range

Biotype / Isolate	Colony type	Fluorescence	Pathogenicity				
			Barley	Oat	Rye	Triticale	Wheat
Barley							
S-80-603	Barley	−	+	−	−	−	−
S-80-611	Barley	−	+	−	−	−	−
S-81-B9	Barley	−	+	−	−	−	−
S-82-13	Barley	−	+	−	−	−	−
S-83-2	Barley	−	+	−	−	−	−
S-83-3	Barley	−	+	−	−	−	−
S-83-6	Barley	−	+	−	−	−	−
S-83-7	Barley	−	+	−	−	−	−
S-83-8	Barley	−	+	−	−	−	−
S-83-9	Barley	−	+	−	−	−	−
S-84-3	Barley	−	+	−	−	−	−
S-92-4	Barley	−	+	−	−	−	−
S-92-5	Barley	−	+	−	−	−	−
S-92-7	Barley	−	+	−	−	−	−
S-93-38	Barley	−	+	−	−	−	−
S-93-39	Barley	−	+	−	−	−	−
Intermediate							
S-84-2	Barley	−	+	−	±[a]	±	±
Wheat							
S-74-20A	Wheat	+	−	−	+	+	+
S-79-4	Wheat	+	+	−	+	+	+
S-80-301	Wheat	+	±	−	±	+	+
S-80-607	Wheat	+	±	−	+	+	+
S-81-W12	Wheat	+	−	−	+	+	+
S-81-B10B	Wheat[b]	+	+	−	+	+	+
S-81-B13B	Wheat (B)[c]	±	±	−	+	+	+
S-82-12	Wheat	+	−	−	+	+	+
S-82-15B	Wheat	+	±	−	+	+	+
S-82-18B	Wheat	+	−	−	+	+	+
S-83-5	Wheat	+	−	−	+	+	+
S-86-1	Wheat	+	−	−	+	+	+
S-89-3	Wheat	+	−	−	+	+	+
S-92-2	Wheat	+	±	−	+	+	+
S-92-8	Wheat	+	±	−	+	+	+
S-92-9	Wheat	±	±	−	+	+	+
S-92-10	Wheat (B)	+	±	−	+	+	+
S-92-11	Wheat	+	±	−	+	+	+

[a] Weak in fluorescence or pathogenicity.
[b] Colony color is dark olive.
[c] Intermediate, has some characteristics of barley colony type.

substitutions per locus that have occurred in the separate evolution of the two populations (1,24). Calculations of these genetic parameters were facilitated by using the computer program BIOSYS-1 (39). This program also calculates these genetic parameters adjusted to small sample sizes as proposed by Nei (25). The sample-size-adjusted genetic distances are also reported.

The second approach is based on discrete characters. Each hybridizing DNA band was considered a character, and each character has two possible states of presence (coded as 1) and absence (coded as 0). In the study comparing the barley and wheat biotypes (study 1), a total of 69 discrete characters were observed among 27 isolates (14 isolates of the barley biotype and 13 of the wheat biotype), and a matrix of 69 × 27 was formed. In study 2, where the two biotypes of *S. nodorum* were compared with *S. a. triticea* and *S. arenaria,* 179 characters were observed among 38 isolates. Thus a second data matrix of 179 × 38 was formed. Both of the two data matrices were independently subjected to cluster analysis. Dice similarity coefficients (S_d) were used to reduce the character data matrices to pair-wise similarity values and were calculated for each pair of the isolates. $S_d = 2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of hybridizing DNA bands shared by the isolates x and y, and N_x and N_y are the numbers of DNA bands in isolates x and y, respectively. The similarity coefficients were used in cluster analysis by using the unweighted-pair-group methods with arithmetic average (UPGMA) clustering algorithm of the computer program NTSYS-pc (31). The second data matrix with 38 isolates of the three species was also used in phylogenetic analysis using maximum parsimony. A hypothetical taxon with all null alleles was created and used as an outgroup (38). Due to the number of isolates involved, only heuristic searches were employed; these include general, stepwise addition (random sequence addition), and branch swapping (tree bisection-reconnection) algorithms. Ten replications of each of the three types of heuristic searches were performed. Bootstrap values (12) were calculated using PAUP (38) to estimate the putative confidence level and support for the internal branches. It is a computationally intensive exercise due to the number of isolates involved. Only 500 replications were performed.

RESULTS

Host range and cultural characteristics. None of the isolates infected oat in the detached leaf inoculations. Typical wheat biotype isolates from wheat caused lesions on wheat, rye, and triticale. Eight isolates of *S. nodorum* from barley were identified as the wheat biotype based on host range and cultural characteristics (8,9). Six of these were isolated from barley seeds, and two were recovered from leaves (Table 2). Eleven of the 18 isolates classified as the wheat biotype were moderately pathogenic to barley (Table 2). Seven wheat biotype isolates were isolated from barley, two from little barley, and two from triticale (Tables 1 and 2). Infection of barley, however, was not limited to wheat biotype isolates from barley. Some typical wheat biotype isolates from wheat also caused symptoms in barley (data not shown). In each instance, lesions expanded more slowly on barley, and the final length of the lesions was about 50% of those on wheat. However, two wheat biotype isolates, S-79-4 from triticale, and S-81-B10B from barley, produced large lesions on barley (Table 2). Two of the wheat biotype isolates from barley, S-81-B13B and S-92-9, were weakly fluorescent on oxgall agar under near-ultraviolet light (Table 2). Isolate S-81-B10B differed from all other isolates in that its colony was dark olive rather than the typical pink to yellow color of wheat biotype colonies on Czapek-Dox V8 agar. Isolates S-92-10 and S-81-B13B had colony morphology that was intermediate between the two biotypes (Table 2).

On Czapek-Dox V8 agar, typical barley biotype isolates were distinctly dark violet with black pycnidia and appressed white mycelium, except at the edge of the colony where more aerial mycelium developed. They were nonfluorescent on oxgall agar and other media (9). Barley biotype isolates were recovered only from barley and were pathogenic to barley but not to wheat, oat, rye, or triticale (Table 2). One isolate from barley, S-84-2, exhibited characteristics intermediate to those typical of the wheat and barley biotypes. S-84-2 had barley biotype cultural characteristics, was nonfluorescent, grew slowly at 31 C, and was pathogenic to barley. However, this isolate also was pathogenic to rye, triticale, and wheat, a character of the wheat biotype.

RFLPs of the barley and wheat biotypes of *S. nodorum*. Twenty-six anonymous DNA probes randomly selected from barley biotype isolate S-83-2 detected 75 alleles among 27 isolates (14 barley biotype isolates and 13 wheat biotype isolates) (Table 3). Two of the 26 probes (SNBE51 and SNBE 95) were monomorphic for the 27 isolates. Eighteen DNA probes detected no variation in the barley biotype and differentiated it from the wheat biotype. Likewise, 17 probes detected no variation in the wheat biotype and differentiated it from the barley biotype (Table 3). DNA probes SNBE 39 and SNBE 56 were specific for the barley biotype and did not detect any DNA bands in the wheat biotype (Table 3). Probe SNBE39 detected eight distinct bands and could be used for DNA fingerprinting of the barley biotype isolates (Table 3). The mean number of alleles per locus, the proportion of polymorphic loci, and the mean gene identity indicated low levels of genetic variation within each of the two populations (Table 3). However, genetic distance suggested that 2.286 allelic substitutions per locus have occurred in separate evolution of the two populations (Table 3). In cluster analysis of the 69 distinct bands, the barley and the wheat biotypes each formed a tight cluster with Dice similarity value above 0.82 (Fig. 1). The clusters based on the DNA bands correlated well with the biotypes determined based on cultural characters and host range (Table 2). Isolate S-84-2, classified as an intermediate biotype (Table 2), had DNA banding patterns identical to the barley biotype and clustered with this group (Fig. 1).

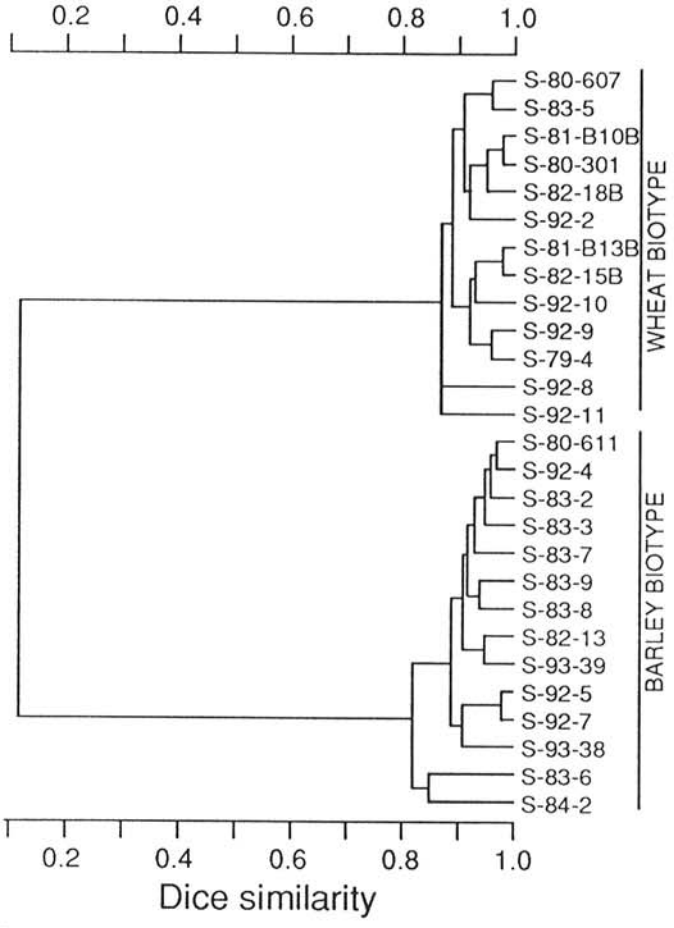

Fig. 1. Cluster analysis of 27 isolates of *Stagonospora nodorum* based on Dice similarity coefficient of 69 individual DNA bands produced by hybridization with 26 anonymous DNA probes randomly selected from a DNA library of barley biotype isolate S-83-2. Cophenetic correlation was 0.998. The two major clusters correspond to the wheat and barley biotypes based on culture characters and pathogenicity.

Relationship of the three *Stagonospora* species. In the hybridization of 38 isolates of the three *Stagonospora* species with 31 DNA probes, the barley biotype is clearly genetically distinct from the wheat biotype. The two biotypes shared only three alleles among the 31 RFLP loci, and the three shared alleles, including a null allele, were detected by probes SNWE9, SNWE72, and SNBE90. Similar to study 1 above where only the two biotypes were compared, low levels of genetic variation were detected within each of the two biotypes (Table 4). On the other hand, *S. a. triticea* exhibited a considerably higher level of genetic variation (Table 4). For instance, the mean gene identity was 0.579 for *S. a. triticea* compared with 0.962 and 0.947 for the wheat and barley biotypes, respectively.

The genetic distance between the wheat biotype and the barley biotype based on the 31 RFLP loci (2.285) (Table 4) was in close agreement with the estimate based on the 26 RFLP loci in study 1 (Table 3). The genetic distance between the wheat biotype and the barley biotype (2.285) was similar to the genetic distance between the wheat biotype and the *S. a. triticea* (2.188) (Table 4). However, the genetic distance between the barley biotype and the *S. a. triticea* (1.603) was smaller than the distance between the barley biotype and the wheat biotype (2.285) (Table 4).

Similarly, the analytic approach based on individual hybridizing bands using cluster and parsimony analyses also supported the relationships of the two biotypes of *S. nodorum* with *S. a. triticea* presented by the genetic distances. The barley biotype had a closer relationship with the *S. a. triticea* than with the wheat biotype (Figs. 2 and 3). High similarity values were shown within each of the two biotypes, and each biotype formed a tight cluster (Figs. 1 and 2). The single isolate of *S. arenaria* showed a closer relationship with two *S. a. triticea* isolates from New York and Washington, and the three isolates formed a loose cluster (at similarity value 0.3) (Fig. 2), but such a relationship was not supported in bootstrap analysis (Fig. 3). All three heuristic searches produced an identical tree (Fig. 3).

The 11 isolates of *S. a. triticea* showed more genetic variation than either of the two biotypes of *S. nodorum* (Table 4, Figs. 2 and 3). The mean number of alleles per locus, the proportion of polymorphic loci, and the mean gene identity for the 11 *S. a. triticea* isolates were 3.52, 1.00, and 0.579, respectively. However, eight of the isolates (seven ATCC cultures originally from Minnesota and one isolate from North Dakota) were genetically similar. Only seven (SNWE26, SNWE64, SNBE47, SNBE55, SNBE67, and SNBE75) of the 31 probes detected polymorphisms

TABLE 3. Comparison of the barley biotype with the wheat biotype of *Stagonospora nodorum* based on allelic frequencies of 26 restriction fragment length polymorphism (RFLP) loci

Probe[a] (size)	Allele[b]	Hybridizing fragments[c] (kb)	Barley biotype (n = 14)		Wheat biotype (n = 13)	
			f[d]	J[e]	f[d]	J[e]
SNBE5	a	1.4	1.000	1	0	1
(1.4)	b	3.8	0		1.000	
SNBE8	a	0.2, 0.5	0.571	0.51	0	0.574
(0.5)	b	0.5	0.429		0	
	c	0.2, 19	0		0.308	
	d	null	0		0.692	
SNBE10	a	0.7	1.000	1	0	1
(0.7)	b	2.0	0		1.000	
SNBE14	a	1.6	1.000	1	0.308	0.362
(1.6)	b	1.7	0		0.462	
	c	null	0		0.231	
SNBE19	a	1.1	1.000	1	0	1
(1.1)	b	20	0		1.000	
SNBE25	a	0.5	1.000	1	0	1
(0.5)	b	5.5	0		1.000	
SNBE34	a	0.8	1.000	1	0	1
(0.8)	b	7.0	0		1.000	
SNBE35	a	1	1.000	1	0	0.526
(1.0)	b	2.8	0		0.385	
	c	null	0		0.615	
SNBE38	a	0.8	1.000	1	0	0.739
(0.8)	b	25	0		0.846	
	c	4.8	0		0.154	
SNBE39	a	5, 10	0.071	0.132	0	1
(0.6)	b	0.6, 2.7	0.071		0	
	c	2, 15, 25	0.071		0	
	d	0.6, 2.7, 15	0.143		0	
	e	0.6, 2.7, 15, 19	0.214		0	
	f	0.6, 2.7, 10, 15, 25	0.071		0	
	g	0.6, 2.7, 15, 19, 25	0.071		0	
	h	0.6, 2.7, 10, 15, 19	0.143		0	
	i	0.6, 2.7, 10, 15, 19, 25	0.143		0	
	j	null	0		1.000	
SNBE47	a	0.6, 15	1.000	1	0	1
(0.6)	b	2.3, 18	0		1.000	
SNBE51	a	0.6	1.000	1	1.000	1
(0.6)						

(*continued on next page*)

[a]Derived from cloned fragments of genomic DNA from barley biotype isolate S-83-2 after digestion with *Eco*RI restriction enzyme. Size is in kb.
[b]Each allele is defined by a set of hybridizing DNA bands of that putative locus.
[c]Null indicates that no DNA bands were detected by that particular probe (null allele).
[d]Frequency of the allele in the population.
[e]Gene identity (J) is the probability that two randomly chosen alleles from the population will be identical (the total of the squared frequencies of all alleles of a particular locus found in the population).

among these eight isolates. The mean alleles per locus, the proportion of polymorphic loci, and the mean gene identity for the eight isolates were 1.32, 0.26, and 0.919, respectively. If the eight isolates were used to represent *S. a. triticea* in comparison with the two biotypes of *S. nodorum,* the sample-size-adjusted genetic distances were 2.673 between the wheat biotype and the eight isolates and 1.979 between the barley biotype and the eight isolates of *S. a. triticea* (calculations not shown). In cluster analysis, these eight isolates formed a tight cluster with an average similarity value of 0.91 (Fig. 2). The remaining three isolates were distantly related to each other and to the Minnesota cluster (Fig. 2). The genetic deviation among S-81-W10, SAT002NY-84, and those *S. a. triticea* isolates from Minnesota and North Dakota was reported earlier (42) and was confirmed here with probes from the barley biotype isolate (Table 4, Fig. 2). ATCC26377 was quite different from the majority of the *S. a. triticea* isolates. It would not hybridize with probes SNWE26, SAAE37, SNBE34, and SNBE75, as could the other *S. a. triticea* isolates. They shared the same DNA banding patterns in RFLPs with only two probes (SNWE40 and SAAE15). ATCC26377 showed a closer relationship with the wheat biotype in phylogenetic analysis, but this branch had a low bootstrap value (Fig. 3). Three tight clusters were formed by the isolates of the wheat biotype, the barley biotype, and the eight isolates of *S. a. triticea* (Fig. 2), and these three clusters were strongly (100%) supported in bootstrap analysis (Fig. 3).

DISCUSSION

S. nodorum is delineated based on the morphology of asexual spores and therefore may not constitute a natural grouping of isolates. It has wheat- and barley-adapted biotypes that are differentiated by pathogenicity and cultural characters (9,15,27,35). Data presented here demonstrate that the two biotypes have distinct RFLPs and form tight, distantly related clusters (Fig. 1). The variation in restriction fragment banding patterns correlates well with the biotypes as determined by biological characters (Table 2). When the genetic variation was compared with isolates of *S. a. triticea,* the barley biotype of *S. nodorum* had lower genetic distance and higher similarity value with the *S. a. triticea* than with the wheat biotype of *S. nodorum* (Table 4, Figs. 2 and 3). Genetic distance measures the accumulated number of allelic substitutions per locus that have occurred in the separate evolution of two populations (1,24). Assuming equal rate of

TABLE 3. (*continued from preceding page*)

Probe[a] (size)	Allele[b]	Hybridizing fragments[c] (kb)	Barley biotype (n = 14) f[d]	Barley biotype (n = 14) J[e]	Wheat biotype (n = 13) f[d]	Wheat biotype (n = 13) J[e]
SNBE55	a	1	1.000	1	0	1
(1.0)	b	2.3	0		1.000	
SNBE56	a	1.1	0.286	0.418	0	1
(1.1)	b	1.4	0.571		0	
	c	1.4, 2.3	0.071		0	
	d	2.3	0.071		0	
	e	null	0		1.000	
SNBE57	a	1.6	1.000	1	0	1
(1.6)	b	19	0		1.000	
SNBE65	a	1.2	1.000	1	0	0.574
(1.2)	b	45	0		0.692	
	c	30	0		0.308	
SNBE66	a	0.9	1.000	1	0	1
(0.9)	b	null	0		1.000	
SNBE67	a	1.4	1.000	1	0	1
(1.4)	b	0.5, 6.5	0		1.000	
SNBE68	a	0.6	1.000	1	0	0.574
(0.6)	b	45	0		0.308	
	c	18	0		0.692	
SNBE71	a	0.4, 1.1	0.857	0.745	0	1
(1.1)	b	16	0		1.000	
	c	0.4, 1.1, 2.3	0.071		0	
	d	1.1, 2.3	0.071		0	
SNBE75	a	1.7	1.000	1	0	1
(1.7)	b	1.3, 4.5	0		1.000	
SNBE76	a	1.3, 16	0.714	0.592	0	1
(1.3)	b	16, 21	0		1.000	
	c	1.2, 16	0.286		0	
SNBE95	a	1.3	1.000	1	1.000	1
(1.3)						
SNBE106	a	0.7	1.000	1	0	0.526
(0.7)	b	25	0		0.615	
	c	9	0		0.385	
SNBE112	a	0.7	1.000	1	0	1
(0.7)	b	6.5	0		1.000	
SNBE127	a	0.7	0.500	0.388	0	1
(0.7)	b	3.5	0		1.000	
	c	8	0.357		0	
	d	0.7, 8	0.071		0	
	e	null	0.071		0	
Mean number of alleles per locus			1.69		1.31	
Proportion of polymorphic loci			0.23		0.27	
Mean gene identity (J_m) (standard error)			0.876 (0.059)		0.880 (0.042)	
Genetic distance (D) between barley and wheat biotypes (D adjusted for sample sizes)				2.292 (2.286)		

evolution in both *S. nodorum* and *S. a. triticea,* it is inferred that the separation of the two biotypes of *S. nodorum* preceded or at least occurred at the same evolutionary time as the separation of *S. nodorum* and *S. a. triticea.*

The RFLP data were analyzed by using two independent approaches: the approach based on allelic frequencies to calculate gene identity and genetic distance, and the approach based on discrete hybridizing bands in cluster and phylogenetic analyses. The outcomes of the two analytic approaches were in agreement. In the approach based on discrete bands, clusters formed by the isolates in cluster analysis were also generally confirmed by phylogenetic analysis. The agreement in the outcomes of the two analytical approaches provided more confidence in interpreting the experimental results.

The results from the host range test and the cultural characters evaluation provided additional evidence that *S. nodorum* exists as two distinct biotypes. The barley biotype is more restricted in host range and was isolated only from barley. Isolates with characteristics of the wheat biotype were isolated from wheat, triticale, ryegrass, little barley, and barley. This agrees with results from previous extensive surveys of wheat and barley seeds. The wheat biotype was often recovered from barley seeds, but the barley biotype was not isolated from wheat seeds (8,21). Other investigators also recovered isolates of the wheat biotype from barley (16,28). None of the *S. nodorum* isolates in this study infected oat in the detached leaf inoculations, which differs from the report by Johnston and Scott (16).

Isolates that fit the basic pathogenicity pattern of the two biotypes of *S. nodorum* may differ in one or more of the secondary characters. Slight differences in morphology and biological characters between the two biotypes are not always clear-cut. Newton and Caten (28) found four out of 64 isolates that had characteristics of both the wheat and barley biotypes. In the present study, one isolate, S-84-2, showed characteristics of both biotypes, but DNA typing placed it within the barley biotype (Table 1, Fig. 1). There were also variations in the intensity of culture fluorescence and colony morphology in some isolates (Table 2).

The existence of two biotypes of *S. nodorum* that differ in host range and biological characters is similar to the situation encountered with *Pseudocercosporella herpotrichoides* (Fron) Deighton, which causes the eyespot disease of cereals. The fungus occurs as a biotype adapted to wheat and barley (W-type), and as a biotype adapted to wheat, barley, and rye (R-type) (5). Each type has its own cultural and physiological characters, but these characters overlap enough to make positive identification difficult (40). RFLP analysis has been used successfully to differentiate the two biotypes (14,40). This is another example of distinct phenotypic and genetic variability in populations of fungi that are identical based on the conidial morphology used in classical taxonomy.

Wheat and barley biotype isolates of *S. nodorum,* which reportedly had considerable karyotype variation (4), can be differentiated from each other based on RFLPs. Among 26 randomly chosen probes developed from a barley biotype isolate, SNBE39 and SNBE56 can specifically identify barley biotype isolates without ambiguity (Table 3). Twelve probes detected monomorphic but distinct RFLP alleles in each biotype. Two sets of

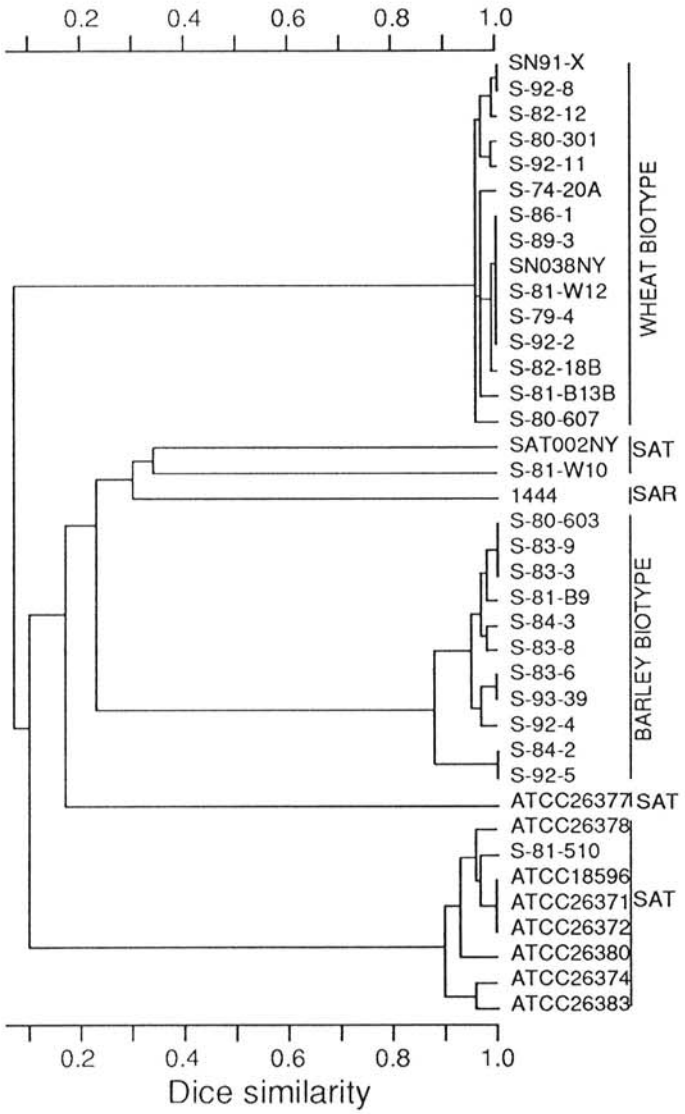

Fig. 2. Cluster analysis of 38 isolates of *Stagonospora nodorum* (two biotypes), *S. avenae* f. sp. *triticea* (SAT), and *S. arenaria* (SAR) based on Dice similarity of 179 individual DNA bands produced by hybridization with 11 DNA probes from the wheat biotype, 12 probes from the barley biotype, and eight probes from *S. a. triticea.* Cophenetic correlation was 0.997.

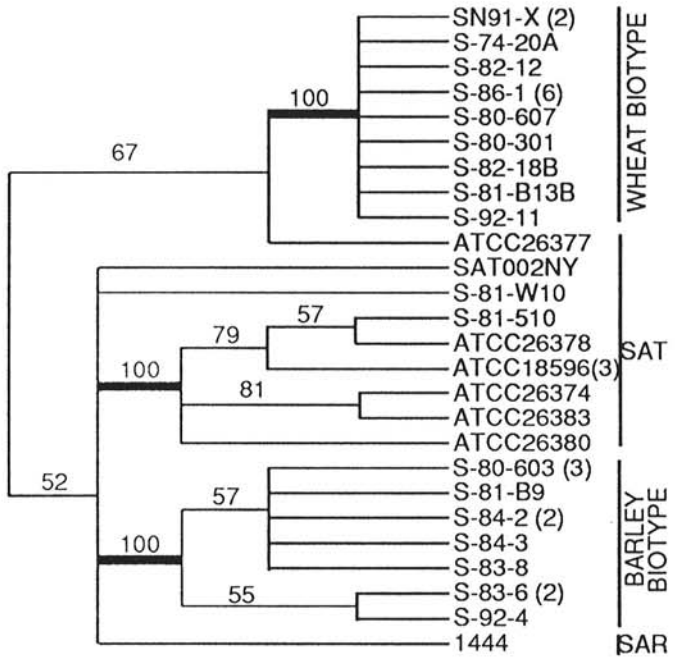

Fig. 3. Phylogenetic relationship of 38 isolates of *Stagonospora nodorum* (two biotypes), *S. avenae* f. sp. *triticea* (SAT), and *S. arenaria* (SAR) generated using PAUP (Phylogenetic Analysis Using Parsimony) based on 179 individual hybridizing DNA bands using maximum parsimony. Bootstrap values of 500 replications of the internal branches are indicated. Branches supported by less than 50% were collapsed to yield polytomies. The number of isolates that had identical RFLP banding patterns for all 31 probes (Dice similarity = 1) (Fig. 2) are indicated in parentheses after the isolate number.

five probes revealed genetic variation individually within each biotype. Nevertheless, genetic variation as measured by the proportion of polymorphic loci and mean gene identity within both biotypes was low. However, the genetic distance between the two biotypes (2.285) was considerable higher than reported for other groups of organisms. Genetic distances between species usually range from 0.8 to 1 in plants and reptiles (1).

Based on gametic equilibrium in *S. nodorum* on wheat, McDonald et al (23) speculated that ascospores of the teleomorph are functional in the field. The low amount of genomic variation in the barley biotype of *S. nodorum* may be related to strong selection pressure exerted by the host or a limited role of the sexual stage in the life cycle. Smedegard-Petersen (35) found both the anamorph and the teleomorph of the barley biotype in Denmark, but it has not been found in the southeastern United States or other locations. Likewise, the teleomorph of the wheat biotype has not been found in the southeastern United States (B. Cunfer, *personal observation*). These results are similar to those reported for the wheat biotype of *S. nodorum* and isolates of *S. avenae* and *S. a. triticea* (41,42).

Among the isolates used in this study, the wheat biotype, the barley biotype, and eight isolates of *S. a. triticea* could be easily recognized by high similarity values in cluster analysis (Fig. 2) and high bootstrap values in phylogenetic analyses (branches indicated by bold lines in Figure 3). The taxonomical affinities of the remaining three isolates of *S. a. triticea* and the isolate of *S. arenaria* could not be determined. Although a loose cluster was formed by isolates SAT002NY and S-81-W10 of *S. a. triticea* and the isolate of *S. arenaria,* it was not supported in phylogenetic analysis (Fig. 3). This indicates that clustering based on low similarity values may not be reliable.

Several probes detected alleles that are specific either for the barley biotype or for the wheat biotype (Tables 3 and 4). Some probes can be used to identify subsets of the barley biotype (Table 3). Probe SNBE71 identified isolates S-83-7 and S-83-8 of the barley biotype from Holland, Virginia (data not shown). It has been suggested that the passage of one biotype of *S. nodorum* through the alternative host several times by artificial inoculation and reisolation increased the pathogenicity of that biotype toward the alternative host (13,32). However, whether the increment of virulence after host passage was due to inoculum contamination (29) or genetic alteration (3,6,33,34) was not determined and remains a controversy. The development of specific probes to identify either biotype should be useful in clarifying this problem.

TABLE 4. Comparison of the wheat and barley biotypes of *Stagonospora nodorum* with *S. avenae* f. sp. *triticea* (SAT) based on allelic frequencies of 31 restriction fragment length polymorphism (RFLP) loci

Probe number[a]	Probe size (kb)	No. of alleles[b]	Gene identity (J)[c] Wheat biotype (n = 15)	Barley biotype (n = 11)	SAT (n = 11)
SNWE9	1.2	2	1	1	0.835
SNWE10	2.2	3	1	1	0.570
SNWE26[d]	1.4	6	1	1	0.438
SNWE30[d]	1.4	6	1	1	0.554
SNWE40[d]	1.4	5	1	1	0.686
SNWE48[d]	1.7	6	1	1	0.554
SNWE64	1.4	6	1	1	0.273
SNWE71[d]	1.1	4	1	1	0.570
SNWE72	1.3	2	1	1	0.702
SNWE85	1.4	6	1	0.488	0.686
SNWE120[d]	1.6	6	1	1	0.554
SAAE7[d]	0.5	6	0.886	0.702	0.835
SAAE15	0.3	6	1	1	0.702
SAAE19	0.8	6	0.680	1	0.554
SAAE24	0.4	2	1	1	0.835
SAAE37	1.0	6	0.502	1	0.570
SAAE52[d]	0.9	7	1	0.686	0.554
SAAE86	0.6	7	0.876	0.702	0.554
SAAE90	1.3	7	1	1	0.686
SNBE5[d]	1.4	8	1	1	0.283
SNBE10[d]	0.7	5	1	1	0.570
SNBE19	1.1	3	1	1	0.835
SNBE25[d]	0.51	5	1	1	0.570
SNBE34[d]	0.8	5	1	1	0.570
SNBE47	0.6	9	0.876	0.835	0.438
SNBE55[d]	1.0	5	1	1	0.372
SNBE57	1.6	4	1	1	0.554
SNBE66	0.9	6	1	1	0.554
SNBE67[d]	1.4	6	1	1	0.355
SNBE75	1.7	5	1	1	0.570
SNBE112[d]	0.7	5	1	1	0.554
Mean number of alleles per locus			1.23	1.23	3.52
Proportion of polymorphic loci			0.19	0.16	1.00
Mean gene identity (J_m)			0.961	0.948	0.578
(standard error of J_m)			(0.011)	(0.015)	(0.020)
Genetic distances (D adjusted for sample sizes)					
Wheat biotype vs. barley biotype				2.287 (2.285)	
Wheat biotype vs. SAT				2.207 (2.188)	
Barley biotype vs. SAT				1.622 (1.603)	

[a]The SNWE series probes were derived from isolate SN209NY-88 of wheat biotype, the SAAE series probes from isolate SAA001NY-85 of *S. a. triticea*, and the SNBE series probes from isolate S-83-2 of barley biotype.

[b]The number of alleles detected by the RFLP probe among the three populations. Each restriction fragment or set of restriction fragments detected by an RFLP probe was assumed to be an allele for that putative RFLP locus.

[c]Gene identity (J) is the probability that two randomly chosen alleles from the population will be identical (the total of the squared frequencies of all alleles of a particular locus found in the population).

[d]Probes detected specific but different alleles for the wheat and barley biotypes.

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