Spatial Diversity of *Setosphaeria turcica* Sampled from the Eastern United States

Lisa M. Ferguson and M. L. Carson

First author: Department of Plant Pathology, North Carolina State University, Raleigh; and second author: U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) Plant Science Research, Raleigh, NC.

Current address of M. L. Carson: USDA-ARS Cereal Disease Laboratory, St. Paul, MN 55108.

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ABSTRACT

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Randomly amplified polymorphic DNA (RAPD) markers and mating type were used to examine regional population structure of *Setosphaeria turcica* in the eastern United States. Of 251 maize-infecting isolates studied, 155 multilocus haplotypes were identified using 21 RAPD markers. Twelve isolates of the most common haplotype were identified from seven states and represented 5.2% of the sample. Although variation in genetic diversity was greatest within states rather than between either regions or states within regions, multidimensional scaling based on average

taxonomic distances among state samples showed a close association of samples from IL, OH, IN, IA, MN, MI/WI, and NC. Isolates from GA/SC, VA/TN, PA/NY, and FL were distant from this core group that included midwestern states and NC and were distinct from one another. The high genotypic diversity, near equal mating type frequencies, and gametic phase equilibrium in samples from several states are inconsistent with a strictly clonal population. The population genetic structure of *S. turcica* is likely the result of both asexual and sexual reproduction. It is not clear whether sexual recombination actually occurs in the eastern United States or occurs elsewhere in tropical America and recombinant genotypes migrate to North America.

Setosphaeria turcica (Luttrell) K.J. Leonard & E.G. Suggs (anamorph = *Exserohilum turcicum* (Pass.) K.J. Leonard & E.G. Suggs) is an ascomycete formerly called *Helminthosporium turcicum* (23) and is the cause of northern leaf blight (NLB) of maize (*Zea mays* L.). *S. turcica* can cause severe disease in the United States and elsewhere in the world, particularly where temperatures during the growing season are moderate (15 to 25°C) and dew heavy (25). Crop losses may exceed 50% if infection becomes severe prior to flowering (35,43).

The use of resistant cultivars is the most effective and prevalent means of controlling NLB (36,47). The use of qualitative sources of resistance, such as *Ht* genes, to control NLB has several limitations. Several pathogenic races of *S. turcica* have been reported, based on their avirulence/virulence to the major resistance genes *Ht1*, *Ht2*, *Ht3*, and *Htn1* (*HtN*) (22). Qualitative sources of resistance such as these *Ht* genes have been supplanted by quantitative (polygenic) sources of resistance (36,47).

The degree of genetic diversity in *S. turcica*, aside from race, has not been extensively examined in the United States. Simcox et al. (38) investigated isozyme diversity among different races of *S. turcica* sampled from the U.S. Corn Belt. Eight electrophoretic isozyme phenotypes were identified out of 40 isolates sampled. Their focus was to identify phenotypes associated with race specificity, but they also were able to associate geographic location with two unique isozyme polymorphisms. However, many isolates sharing common isozyme electrophoretic phenotypes were from widely dispersed locations in the United States. The sexual stage of *S. turcica* has not been found in the field; although there

is evidence that recombination is occurring, particularly in tropical populations (3,5). Co-existing compatible mating types have been found in the field (1,3,33). Matings of compatible isolates can be induced easily in the laboratory, supporting the possibility that the sexual cycle may play a role in the organism's survival in nature (3,11,21).

Studies of population structure of plant-pathogenic fungi may help to assess a pathogen's potential to adapt to host resistance or environmental changes (24). In addition, such studies may provide support for resistance breeding programs and deployment strategies that improve disease control. Our goals in this study were to examine genetic diversity in collections of *S. turcica* from different geographic locations throughout the eastern United States using random amplified polymorphism DNA (RAPD) markers.

Mating type frequencies also were studied by location of origin. Specifically, we estimated genotypic and gene diversity in samples of *S. turcica* collected from different states in the eastern United States. Virulence survey collections made by DeKalb Genetics (DeKalb, IL) were used as a representative sampling of North American *S. turcica* populations. These samples were collected during the maize-growing season at field locations in 19 eastern states. Additional samples were also examined as part of the study collection.

MATERIALS AND METHODS

Isolate collection assembly. The collection consisted of 261 *S. turcica* isolates, including three isolates from *Sorghum halepense* and three from *Sorghum bicolor* in addition to the maize isolates. Isolates of *S. turcica* were obtained from several sources. The majority of the isolates (193 total) were sampled by DeKalb Genetics in yearly surveys of maize foliar pathogens infecting 25 maize inbred lines (39,40). Samples were collected for isolation, identification, and testing of isolates on differential inbred lines.

Corresponding author: M. L. Carson; E-mail address: mcarson@umn.edu

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DeKalb Genetics supplied samples of *S. turcica* on dried leaf tissue for our study (J. Kinsey, J. M. Perkins, and D. R. Smith). Infected greenhouse-grown leaf tissue was dried and stored in labeled envelopes at 4°C at their facility in DeKalb, IL. Most isolates were readily recovered in our study. Additional isolates collected in the Midwest (IL, IN, OH, and MN), North Carolina (M. L. Carson), and Florida (J. K. Pataky) were included in our studies. *Sorghum halepense*-infecting isolates were collected in North Carolina (M. L. Carson). Isolates from *Sorghum bicolor* from Indiana were supplied on dried leaf tissue (L. D. Dunkle, USDA-ARS, West Lafayette, IN). Four isolates collected in 1957 from Virginia (C. W. Roane) were also evaluated. The study collection contains isolates of *S. turcica* beginning in 1974 to 1994 from 20 different states (Table 1). Samples were grouped in the analysis according to proximity of collection sites and similarity of cultivation practices.

Isolation of *S. turcica* from leaf tissue. Three characteristic NLB lesions per sample of leaf tissue were excised and surface-sterilized. Greenhouse-produced leaf tissue was rinsed for 1 min in 0.5% sodium hypochlorite solution followed by rinsing in sterile distilled water for 30 to 60 s. Field-collected tissue was surface-sterilized for 1 min in 70% (vol/vol) ethanol followed by 1 min in 0.5% sodium hypochlorite solution and rinsed in sterile distilled water for 30 to 60 s prior to incubation. Surface-sterilized tissue was incubated on moist sterile filter paper in petri dishes for 2 to 3 days at 20 to 25°C under 12-h fluorescent lighting. Most isolates were readily recovered from dried leaf samples.

Conidia from sporulating lesions were incubated on water agar plates for 24 h to obtain two single-spore isolates from separate lesions for each leaf sample. Single germinated conidia of *S. turcica* were transferred to lactose casein hydrolysate agar (LCA) (44). Cultures grew under 12 h lighting for 10 to 14 days at 20°C until abundant conidia were produced. Conidia of each isolate were harvested in sterile 15% (vol/vol) glycerol solution, placed in duplicate labeled vials, and stored at -80°C for future use.

Race determinations. Isolates obtained from DeKalb Genetics were tested previously to determine race. To confirm the race identity, we assessed avirulence/virulence of isolates on differential inbred lines. Lines used to identify avirulence/virulence were Pa91 (no *Ht* genes), Pa91*Ht*1, Pa91*Ht*2, Pa91*Ht*3, and B68*Htm*1 (formerly *Ht*N). Pots (30.5-cm-diameter, clay) were filled with a 1:1 mixture of Pro-Mix BX steam-sterilized soil (Premier Horticulture Ltd., Dorval, Quebec). All differential lines were planted together in one pot and were inoculated with a single isolate per pot. Three seeds of each differential line were planted in the pot. Two pots containing all five lines were inoculated with each isolate to be race-typed. Plants were grown in the greenhouse at moderate temperatures, 20 to 22°C day/15 to 18°C nights, with supplemental lighting at 25 and 50 klux (325 to 650 $\mu\text{E m}^{-2} \text{s}^{-1}$) average photosynthetic photon flux density, consistent with conditions in Leonard et al. (22). Reactions associated with genes *Ht*1, *Ht*2, and *Ht*3 have been shown to be sensitive to varying temperature and light intensity (7,22). Plants were grown for 19 to 21 days and were inoculated after the fourth leaf fully emerged.

Fungal isolates to be race-typed and isolates of *S. turcica* of known race used as controls were grown from first generation subcultures on LCA (44). Cultures were grown for 7 to 10 days at 20 to 25°C under 12-h light/dark regime to promote sporulation. Conidia were dislodged and rinsed from the petri plates with sterile water containing Tween 20 (10 $\mu\text{l liter}^{-1}$). Filtered conidial suspensions were quantified by a hemacytometer and diluted to approximately 1,000 conidia ml^{-1} . Aliquots of 0.1 ml of conidial suspension were pipetted into the leaf whorl of corn seedlings. Inoculated plants were incubated for 16 h at 20 to 22°C overnight in a mist chamber to simulate conditions conducive to infection. After incubation, plants were moved to greenhouse benches and maintained at preinoculation conditions.

Plants were inspected for symptoms of infection on the *Ht* differential lines at 14 and 20 days after inoculation. Lesions, which appeared typically wilted, gray-green, necrotic, and lacking chlorosis, were scored as susceptible reactions (virulent isolate). Symptoms expressed as lesions surrounded by chlorosis with necrosis were scored as resistant reactions (isolate avirulent). Isolates were identified according to the race scheme outlined in Leonard et al. (22).

TABLE 1. Origin by state, location, and year of collection, and number of isolates (*N*) of *Setosphaeria turcica* studied (host of origin was maize unless otherwise indicated)

State	Location	Years	<i>N</i>
FL	Belle Glade	1982, 90	18
GA	Leesburg	1974, 75, 91, 93	9
IA	Dayton	1977, 78, 79, 80	14
IA	Atlantic		4
IA	Spencer	1983	13
IA	North Liberty	1983, 84, 90, 91, 92, 93, 94	9
IL	Adams Co.	1981	1
IL	DeKalb	1974, 75, 76, 81, 87, 91, 92, 93, 94	23
IL	Geneseo	1981	1
IL	Henry Co.	1981	1
IL	Illioopolis	1985, 91, 92, 93	4
IL	Iroquois Co.	1981	1
IL	Marshall Co.	1981	1
IL	Milford	1979	1
IL	Monmouth	1974	1
IL	Peoria	1981	1
IL	Rankin	1981	1
IL	Schuyler Co.	1981	1
IL	St. Clair Co.	1981	1
IL	Stark Co.	1981	1
IL	Thomasboro	1974, 75, 90, 92, 94	8
IL	Urbana	1982	1
IL	Warren	1977	2
IL	Whiteside Co.	1981	1
IL	Woodford Co.	1981	1
IN	Blackford	1980	1
IN	Clark Co.	1980	1
IN	Crawfordsville	1979, 80, 81, 90	7
IN	Jennings Co.	1980	1
IN	Shelby Co.	1981	1
IN	W. Lafayette ^a	1998	3
KS	Garden City	1987, 92	2
KY	Caldwell Co.	1976	1
MI	Mason	1974, 76, 77, 90, 93, 94	10
MN	Redwood Falls	1977, 78, 79	6
MN	Swift Co.	1981	1
MN	Owatonna	1982, 83, 90, 91	10
MN	Olivia	1992, 94	8
MO	Marshall	1981, 89, 91	3
NE	Fremont	1975, 82	3
NC	Marion	1993	1
NC	Mount Olive	1976, 79, 84, 85, 86, 89, 91, 93	15
NC	Salisbury ^b	1994, 97, 98	3
NC	Wake Co.		1
NC	Wilkes Co.		3
NC	Yadkin		4
NY	Geneva	1981	1
OH	Marion	1974, 75, 76, 90, 92	7
OH	Wayne Co.	1980	1
OH	West Jefferson	1976	1
OH	Deshler	1977, 83, 86	5
PA	Manheim	1994	1
PA	Palmyra	1992, 93	2
PA	York	1981	8
SD	Brandon	1994	1
SC	Estill	1974, 76, 86	4
TN	Union City	1976, 79, 84	3
VA	Petersburg	1974, 75	2
VA	Warrenton	1982	11
WI	Menomonie	1974, 81, 86	3
WI	Madison	1990, 94	2

^a *Setosphaeria turcica* isolated from *Sorghum bicolor* (cultivated sorghum).

^b *Setosphaeria turcica* isolates from *Sorghum halepense* (johnsongrass).

Genomic DNA extraction. DNA extraction methods were adapted from DuTeau and Leslie (9). Our modifications included use of a different lysis buffer, phenol/chloroform solution for extraction, and final treatment of extracted samples with RNase. First generation isolates transferred from stock cultures (-80°C storage) to LCA were grown on oatmeal agar (75 g of ground oatmeal plus 15 g of agar per 1 liter of deionized water) for 4 to 6 days at 20°C with 12-h fluorescent lighting. Mycelium was harvested and frozen with liquid nitrogen prior to adding heated lysis buffer (65°C consisting of 10 mM Tris [pH 7.4], 100 mM lithium chloride, 0.5% sodium dodecyl sulfate, 10 mM EDTA, and proteinase K at 0.1 mg/ml).

Following extraction, DNA was precipitated overnight in ammonium acetate and 95% ethanol, spun down, dried briefly in a spin vac, and resuspended in Tris-EDTA buffer (pH 7.4) and treated with RNase ($1\text{ }\mu\text{g }\mu\text{l}^{-1}$). DNA was quantified by visual comparison of at least two dilutions per isolate to λ -DNA standards in 1.0% agarose gels run in $1\times$ Tris-borate-EDTA (TBE) and stained with ethidium bromide. The quality and quantity of DNA was suitable for use in polymerase chain reaction (PCR) with either random or specific primers.

Mating type tests. The sexual cycle of *S. turcica* can be induced in culture by pairing isolates with tester strains on the appropriate substrate and incubating for 3 to 4 weeks. To streamline the identification of mating type of *S. turcica* isolates in our collection, we used a PCR assay with primers developed to investigate mating type genes in a related fungus, *Cochliobolus heterostrophus* (2).

Arie et al. (2) cloned mating type genes of *C. heterostrophus* using PCR primers designed to correspond to unique DNA sequences that flank the mating type genes, called HMG (high mobility group) boxes. Using the two primers they designed according to the flanking sequences, ChHMG1 and ChHMG2 in a simple PCR procedure, generates a 0.3-kb product from *MAT1-2*, but not from *MAT1-1* strains. These primers were tested successfully by Arie et al. (2) on related ascomycetes, *S. turcica* among them. PCR reactions of *S. turcica* genomic DNA with these primers yielded the expected marker with the *MAT1-2* strains, but not with the *MAT1-1* strains (2). ChHMG1 and ChHMG2 were synthesized by Sigma-Genosys (The Woodlands, TX), dissolved in sterile distilled water at 100 μM concentration, and stored at -20°C . Reaction mixtures containing 10 ng of genomic DNA in a total reaction volume of 25 μl were prepared, and PCR assays were conducted as outlined by Arie et al. (2).

We tested the PCR procedure outlined by Arie et al. (2) with isolates of known mating type. Known mating type isolates used were 266a, 268a, and 270a for *MAT1-1* type testers and 265a, 267a, and 269a for *MAT1-2*. The procedure consistently amplified a fragment of approximately 0.3 kb in *MAT1-2* tester isolates, which was not present in *MAT1-1* testers. Ten microliters of each PCR product was subjected to electrophoresis in 1.5% agarose gel in $1\times$ TBE containing ethidium bromide for 4 h at 75 V and documented with thermoprints. All reactions were run with control isolates of each mating type and repeated for each sample to confirm the results. Mating type was scored based on the amplification of a 0.3-kb product. Presence of the fragment corresponded to *MAT1-2*; absence of the fragment corresponded to *MAT1-1*, as confirmed by control isolates of each mating type. In repeated tests, 37 isolates of the 261 isolates were inconclusive for mating type in the PCR assay.

Inconclusive isolates were paired in conventional tests in culture with mating type tester isolates. Tester isolates used were 1a, 28a, 29a, 56a, 266a, and 268a of *MAT1-1* type and 3a, 45a, 102a, 260a, and 265a of *MAT1-2* type. Sterile johnsongrass (*Sorghum halepense*) culms (2 cm) were embedded in the center of 9-cm-diameter petri dishes (11) containing modified Sach's agar (17). First generation subcultures were grown 4 to 6 days on potato dextrose agar. Agar plugs (No. 1 cork borer, 3 mm) from testers

of *MAT1-1* or *MAT1-2* and unknown cultures were placed 1 cm from the *Sorghum halepense* culm opposite each other. Tester isolates were selfed, paired in all combinations with testers of opposite mating type as well as testers of the same mating type to prevent ambiguous results. Successful mating occurred only between testers of opposite mating type. Duplicate pairings of sample isolates of unknown mating type were conducted with three tester isolates of each mating. Petri dishes containing paired isolates were sealed and placed in the dark at 20°C and were examined under a stereoscope at 3 and 4 weeks after plating for the presence of pseudothecia. Pseudothecia are usually visible in fertile combinations after 10 days and ascospores generally mature after 21 days (26). When mating occurred, it was exclusively with a tester of one mating type. A single isolate, 262a, did not pair with strains of either mating type, even when tested with additional isolates. Ascus and ascospore production in the mating combinations were not assessed. Frequencies of *MAT1-1* and *MAT1-2* isolates in samples from individual states were compared to determine the potential for sexual recombination.

RAPD analysis. Extracted DNA of *S. turcica* was amplified using PCR with random decamer primers (49). A total volume of 25 μl was used for RAPD reactions. Components of the reaction mixture were $1\times$ buffer (2.5 μl of stock Promega $10\times$ buffer consisting of 500 mM KCl, 100 mM Tris-HCl, pH 9.0, at 25°C , and 1% Triton X-100), 0.2 mM dNTP, 2.5 μl of primer (10 $\mu\text{g}/\mu\text{l}$ of stock), 1.5 mM MgCl₂, 5 ng of template DNA, and 0.04 units of *Taq* polymerase (Promega, Madison, WI). RAPD-PCR reactions were run in a thermal cycler (PCT-100; MJ Research, Watertown, MA) using the following protocol: (i) denature at 92°C for 30 s, (ii) anneal at 35°C (primer-dependent) for 1 min, (iii) increase to 72°C , at a rate of $1^{\circ}\text{C}/8\text{ s}$ over 5 min, (iv) extension at 72°C for 2 min, (v) repeat 45 cycles (steps 1 to 4), (vi) final extension at 72°C for 7 min, and (vii) hold at 4°C . Alternate annealing temperatures were used depending on given primers selected. PCR products were analyzed by gel electrophoresis in $1\times$ TBE and were visualized with UV and documented as thermoprints.

Decamer primers were obtained from Operon Technologies (Alameda, CA) and the University of British Columbia (Vancouver, Canada). Primers were selected based on primers used in studies of *S. turcica* populations by Borchardt et al. (3) and those used in population studies of the related organism, *C. heterostrophus* (M. L. Carson, unpublished data). Polymorphisms detected were confirmed in reactions with 0.1, 1.0, and 10 ng of template DNA, and those that were consistent over this range of DNA concentrations were included in the analysis. Seven primers, which amplified 21 reproducible and polymorphic markers, were used for PCR amplification of genomic DNA (Table 2). Positive and negative reference control isolates were identified for each polymorphic marker, to improve consistency of reactions. These controls were used routinely in PCRs and on gels for comparison to sample isolates.

Variation in growing conditions related to sample groupings. Due to the variety of climatic zones and cropping strategies employed in different regions of the country, the pathogen is subject to different agroecological environments. Samples collected in the eastern United States were divided into groups on a regional basis. Florida may present a unique environment for *S. turcica*, where sweet corn and maize seed production fields predominate and most production occurs in the winter months (13). Planting dates in Florida range from July through May and harvest stretches from 25 September to 15 June, with a peak period between mid-November and mid-June. Southern states tend to have smaller, scattered fields than midwestern farms and are generally planted earlier in the spring and harvested earlier than northern Corn Belt states (32). Corn production for grain in midwestern states occurs over much broader and contiguous areas than in the South.

Collections from the southeastern United States included samples from North Carolina (NC) and Florida (FL), as well as

grouped samples from Virginia and Tennessee (VA/TN) and Georgia and South Carolina (GA/SC). Collections from mid-western United States were composed of samples from Ohio (OH) and Minnesota (MN) and grouped samples from Indiana and Kentucky (IN/KY); Illinois, Missouri, and Kansas (IL/MO/KS); Iowa, South Dakota, and Nebraska (IA/SD/NE); Michigan and Wisconsin (MI/WI); and Pennsylvania and New York (PA/NY).

Data analysis. A total of 251 isolates were included in the final data analysis. Fragments amplified by the RAPD-PCR procedure showing polymorphism between isolates correspond to one allele at one locus. Other alleles, not amplified, are unknown. Absence of a particular marker fragment does not necessarily indicate an alternate allele. Multilocus haplotypes were based on presence or absence of 21 RAPD loci.

Genotypic diversity based on Shannon index. Shannon genotypic diversity was calculated based on RAPD haplotypes (20). The measure corrects for different sample sizes as described by Sheldon (37). The equation used was $H_S = \{-\sum_i [g_i \ln(g_i)]\}/\ln N$, where g_i is the frequency of the i th haplotype in one sample and N

is the sample size. This type of index accounts for both the abundance of genotypes and their distribution in the population (15). Genotypic diversity estimates for state samples, H_S values, were compared with a simple t test (19,20).

Gene diversity. Nei's average gene diversity over all loci was measured using the index $H_N = \sum_i h_i/L = \sum_i (1 - \sum_a p_a^2)/L$, where h_i is the gene diversity at the i th locus, L is the number of loci sampled, and p_a is the frequency of the a th allele at the locus in question (31). The gene diversity estimate for haploid populations, h_i , is the same as that described for a homozygous autogamous (self-fertilizing) population. The sampling variance of the gene diversity was calculated by the following equation: variance(H_N) = $\sum_i (h_i - H_N)^2 / [(L - 1) \times L]$. Gene diversity estimates for state populations were compared using pairwise t tests (31).

Comparisons between isolates were made based on the Dice similarity coefficient (41). With x and y indicating the two isolates to be compared, the coefficient was calculated as $S_d = 2n_{xy} / (2n_{xy} + n_x + n_y)$, where n_{xy} is the number of fragments possessed by both isolates, n_x represents the markers possessed only by x , and n_y represents the markers possessed only by y (41). The software

TABLE 2. Frequencies of 21 randomly amplified polymorphic DNA (RAPD) bands, two mating types, and four races considered in samples of *Setosphaeria turcica*^a

Primer band ^b	State(s) of origin										
	FL	GA/SC	NC	VA/TN	PA/NY	IL/MO/KS	IA/SD/NE	IN/KY	OH	MN	WI/MI
N	18	13	24	16	12	58	44	12	14	25	15
Op-B11											
1.7 ^c	1	0.62	0.25	0.69	0.67	0.21	0.09	0.25	0.07	0.08	0.13
Op-I12											
0.51	0.06	0.46	0.21	0.5	0.17	0.19	0.25	0.17	0.07	0.16	0.2
0.55	0.89	0.92	0.92	0.88	0.92	0.97	0.93	1	0.93	0.96	1
0.77	1	1	0.83	0.94	1	0.97	1	1	1	1	0.93
1.6	1	1	0.96	1	1	0.98	1	1	1	1	0.87
2.18	1	1	0.96	0.94	0.92	0.98	0.98	1	1	1	0.93
Op-B14											
2.1	0.94	0.85	0.92	0.94	1	0.98	1	1	0.93	0.88	0.93
Ubc100											
2.05	0.61	0.85	0.88	1	1	0.91	0.98	0.83	1	1	1
2.52	0.5	0.38	0.46	0.31	0.17	0.52	0.36	0.42	0.43	0.44	0.27
Ubc23											
0.82	0.61	0.85	0.67	0.88	0.58	0.79	0.86	0.75	0.86	0.6	0.8
1.35	0.94	0.77	0.92	1	1	0.93	0.95	1	0.86	0.88	1
1.75	0	0.15	0.08	0.25	0	0.12	0.09	0	0.14	0.08	0.13
Ubc417											
0.75	0.22	0.46	0.21	0.19	0	0.36	0.45	0.5	0.21	0.36	0.2
1.12	<u>0.89</u>	1	1	1	1	1	1	1	1	1	1
1.52	1	0.85	0.88	1	1	0.98	0.93	0.83	1	0.96	0.93
1.75	0.44	0.92	0.71	0.5	0.58	0.52	0.36	0.67	0.79	0.56	0.67
Ubc77											
0.65	1	1	0.92	0.94	1	0.98	0.98	1	1	1	1
1.25	1	0.92	0.96	0.95	1	1	1	1	1	1	1
1.45	0.83	0.85	0.83	0.81	0.92	0.84	0.98	0.92	0.71	0.92	0.93
1.6	0.56	0.38	0.5	0.5	0.67	0.67	0.57	0.75	0.43	0.6	0.6
1.7	0.94	0.85	0.92	0.81	0.92	0.93	0.98	0.92	1	1	1
Non-RAPD markers											
MAT1-1 ^d	0.83	0.77	0.46	0.13	0.08	0.4	0.2	0.67	0.5	0.28	0.27
MAT1-2	0.17	0.23	0.46	0.87	0.92	0.6	0.8	0.33	0.5	0.72	0.73
Unknown			0.04								
Race 0 ^e	0.5	0.54	0.38	0.31	0.25	0.52	0.57	0.58	0.79	0.56	0.6
Race 1	0.5	0.15	0.46	0.63	0.75	0.12	0.18	0.17	0.21	0.36	0.27
Race 23	0	0	0.04	0	0	0.12	0.07	0	0	0	0.07
Race 23N	0	0.31	0.13	0.06	0	0.22	0.18	0.25	0	0.08	0.07
Other	0	0	0	0	0	0.02	0	0	0	0	0

^a Frequencies of bands showing polymorphism in only one state sample ("private" alleles) are underlined.

^b Primers are indicated in bold. Primer numbers preceded by Op are from Operon Technologies (Alameda, CA). Numbers preceded by Ubc were obtained from the University of British Columbia, Vancouver.

^c Fragment sizes are compared with DNA size markers in kilobases.

^d Mating type of *Setosphaeria turcica* was determined by polymerase chain reaction and confirmed by in vitro pairings.

^e Race designations are based on reactions to *Ht* resistance genes in maize according to Leonard et al. (22).

package NTSYS-pc (Exeter Software, Setauket, NY) was used to calculate Dice coefficients. Pairwise distances were derived from Dice, where D was calculated from the similarity measure, S_d , as $D = 1 - S_d$ (42).

Modified analysis of variance was performed based on the resulting matrix of squared-distances among all pairs of haplotypes and was used to partition variation within and among different samples of *S. turcica* (10). Permutations-randomizations of the data set at different hierarchical levels allowed significance tests of the distribution of variation. This analysis was conducted using Arlequin version 2.000 software (University of Geneva, Switzerland). Genetic subdivision of the midwestern (seven state populations: IA/SD/NE, IL/MO/KS, OH, IN/KY, MI/WI, MN, and PA/NY) versus the southeastern (four state populations; FL, GA/SC, NC, and VA/TN) isolates was tested.

Using a simple hierarchical genetic structure composed of haploid individuals, the procedure also computed a fixation index, F_{ST} , which is equivalent to the weighted average F statistic over loci, defined by Weir and Cockerham (46) as θ_w . Significance testing of fixation indices was conducted by using a nonparametric permutation approach (10). Permutation of haplotypes, individuals, subpopulations, or populations is used to obtain a null distribution of indices. Under the null distribution, there is no difference between the permuted and observed variation at the hierarchical levels tested (10,45,46). Population pairwise F_{ST} values were calculated to test for significant subdivision at the state level.

The geometric distance parameter, average taxonomic distance (41), was calculated as $d_i = \sqrt{[1/n \sum_l (p_{as} - p_{at})^2]}$. Two sample isolates, s and t , are compared across l loci. The variables p_{as} and p_{at} represent the relative frequencies of the a th allele in isolates s and t . The program modules MDSCALE, MST, and MOD3D of the NTSYS-pc software package were used to visualize distances between samples by employing methods of multidimensional scaling and generating a superimposed minimum-spanning tree.

Index of association. To assess the importance of sexual recombination, the index of multilocus association, I_A , was calculated (6). This index is calculated as $I_A = (V_O/V_E) - 1$, where V_O is the observed and V_E is the expected variance of k . The variable k is the number of loci at which two isolates differ, computed for all possible pairs of isolates; $n(n - 1)/2$. We established a null hy-

pothesis that there was no association of alleles at different loci ($H_O: I_A = 0$), which is consistent with random mating in a population. This hypothesis was tested with an upper confidence limit of V_O , the observed variance of k , which is calculated using the error variance of V_E . The data set was clone-corrected in two ways, considering only one isolate per clone based on multilocus RAPD haplotypes and RAPD plus mating type haplotypes.

RESULTS

RAPD markers. Of the 251 maize-infecting isolates, there were 155 multilocus haplotypes based on 21 RAPD markers. Midwestern samples from IA/SD/NE, IL/MO/KS, IN/KY, OH, MI/WI, and MN shared more haplotypes with one another than they did with sample groups from FL, GA/SC, or VA/TN. There were more shared haplotypes among NC and IA/SD/NE, IL/MO/KS, OH, MI/WI, and MN than there were with southeastern regional group states (FL, GA/SC, and VA/TN). PA/NY shared the greatest number of haplotypes with IL and MI/WI. A few haplotypes recurred over great geographic distances in the U.S. population of *S. turcica*.

The most common RAPD haplotype in the eastern United States was 40, representing 5.2% of the maize-infecting isolates. It was found in IA/SD/NE, MN, NC, IL/MO/KS, MI/WI, OH, and PA/NY. This haplotype differed by only a single marker band from both haplotypes 39 and 36, which were also shared among state groups. The second most common haplotype was 62, representing 3.6% of isolates. This haplotype differed from haplotype 40 by only two RAPD bands. RAPD haplotype 62 was found in IA/SD/NE, IL/MO/KS, MN, and NC. Haplotypes 29 and 66 each were represented by seven isolates and haplotypes 35 and 65 by six isolates. Fifteen other haplotypes were recovered three to five times in the collection. An additional 15 RAPD haplotypes each were found in two isolates. Of 155 total RAPD haplotypes, 119 were represented only by one isolate.

If the collection is divided into Southeast versus Midwest samples, 11 of 155 RAPD haplotypes are shared by both regional samples. Samples from IL/MO/KS, IA/SD/NE, IN/KY, MN, MI/WI, OH, and PA/NY accounted for 180 isolates of 251 total isolates. There were 103 distinct RAPD haplotypes among these isolates. Haplotype 40 remained the most common (6.1%) followed by 62 (4.4%), 66 and 29 (both at 3.9%), and 65 and 35 (both at 2.8%). These haplotypes are overrepresented in samples from the midwestern states.

Restricting the collection to only southeastern samples, represented by the state samples from FL, NC, GA/SC, and VA/TN, reduced the number of isolates to 71. There were a total of 64 RAPD haplotypes, 57 represented by a single isolate. Seven haplotypes (40, 44, 113, 128, 133, 141, and 157) were represented by two isolates only (2.8% each). Of these common haplotypes, all but haplotypes 128 and 141 were also found in the midwestern states. Many haplotypes were restricted to single state samples; however, several haplotypes were present in many states in the United States. There was only a single example of a "private" allele that was either exclusively present or absent in regional or state samples (Table 2).

A multidimensional scaling plot based on average taxonomic distances among state samples showed close association of samples from IL/MO/KS, OH, IN/KY, IA/SD/NE, MN, MI/WI, and NC (Fig. 1). Samples from IL/MO/KS overlapped with samples from MN. Isolates from GA/SC, VA/TN, PA/NY, and FL were distant from the core group that included midwestern states and NC and were distinct from one another. RAPD allele frequencies of GA/SC were most similar to those of VA/TN, although VA/TN was most similar to PA/NY isolates. FL proved to be the most distant from the core group and the remaining states, but was most similar to PA/NY of all state samples, although very distant both genetically and geographically.

Fig. 1. Multidimensional scaling plot of *Setosphaeria turcica* state samples. Distances among state collections plotted in three dimensions are monotone to the original distances (transformed from Dice similarity) calculated by the average taxonomic distance parameter. Lines connect collections using a superimposed minimum spanning tree.

When a hierarchical analysis of molecular variance was conducted, the proportion of the “among regions” variance component was small (6.45%), as was the “among populations within regions” variance component (5.94%) in comparison to the “within populations” variance component (87.61%) (Table 3). All three variance components are significant, indicating a partitioning of genetic diversity at the regional, population, and within population level (Table 3).

Pairwise differences (Table 4) indicated partitioning of molecular variance among state populations. Samples originally included in the Southeast and Midwest regions may be grouped more appropriately based on our data. Samples from IA/SD/NE, IL/MO/KS, IN/KY, MI/WI, MN, and NC did not differ in molecular variance. OH differed in molecular variance only from IA/SD/NE and PA/NY in the midwestern group.

NC differed in molecular variance only from FL among all state samples. FL differed from all groups but PA/NY. The PA/NY sample differed from all groups but IN/KY, FL, NC, and VA/TN. GA/SC was distinct from FL and the midwestern states, with the exception of IN/KY and OH. Molecular variance inherent in the VA/TN sample was significantly different from all state samples but PA/NY and GA/SC.

Mating type. Distributions of *MAT1-1* and *MAT1-2* isolates in samples from individual states were compared to determine the potential for sexual recombination. Mating type frequencies in samples were found in near-equal proportions in NC, OH, IL/MO/KS, and IN/KY. In other states, one mating type was more prevalent than the other. Among state groups with unequal mating type proportions, IA/SD/NE, MI/WI, MN, VA/TN, and PA/NY had a higher proportion of *MAT1-2* isolates. Isolates from FL and GA/SC were predominantly *MAT1-1*.

Gene and genotypic diversity. Genotypic diversities (corrected for sample size) compared by pairwise *t* tests for all states were not significantly different from one another. We were unable to reject the null hypothesis that diversities were equal ($P \leq 0.05$). However, there were numerical differences among states. Samples from IL/MO/KS, IA/SD/NE, and MN had the lowest genotypic diversity (Table 5). PA/NY and FL showed similar and slightly higher genotypic diversity than that of IL/MO/KS, IA/SD/NE, and MN. Collections from VA/TN, GA/SC, MI/WI,

OH, IN/KY, and NC were found to have genotypic diversities at or very near the maximum. Based on Brown's index of multilocus association, IN/KY, OH, MI/WI, MN, PA/NY, FL, and NC populations were near gametic phase equilibrium (Table 5). GA/SC and VA/TN showed strong disequilibrium, and IA/SD/NE and IL/MO/KS showed weak disequilibrium. Nei's average gene diversity was not significantly different among state groups. Numerically higher gene diversities were calculated for GA/SC, NC, and VA/TN. The lowest level of gene diversity was found in PA/NY.

Race determinations. Four races of *S. turcica* were found in the collection. In three state groups, FL, PA/NY, and OH, only races 0 and 1 were present. Race 23 was absent from GA/SC, VA/TN, IN/KY, and MN. In most states, race 0 (avirulent on *Ht* genes 1, 2, 3, and N) predominated. These states were GA/SC, IL/MO/KS, IA/SD/NE, IN/KY, OH, MN, and WI/MI. In three other states (NC, VA/TN, and PA/NY), race 1 (specifically virulent on *Ht1*) was most abundant. FL had equal proportions of race 1 and 0 isolates. Race 23 isolates were found in NC, IL/MO/KS, IA/SD/NE, and MI/WI at frequencies of 0.04 to 0.12. Race 23N isolates were found in all states except FL, PA/NY, and OH. Frequencies of race 23N ranged from 0.06 to 0.31 in states where it occurred.

DISCUSSION

The lack of significant differences in genotypic diversity among state samples is likely due to a lack of subdivision among state populations in the eastern United States. High genotypic diversity in the samples studied provides evidence that sexual recombination plays a role in U.S. populations of *S. turcica*. Based on our studies, it is unlikely that *S. turcica* populations are strictly clonal. It is more reasonable that they consist of a mixture of individuals derived from both asexual and sexual reproduction. Measurement of gametic phase disequilibrium by the index of association allows inferences to be made about the occurrence of sexual reproduction in populations (6,30,48), although it is not independent of allele frequencies (18). Samples from IA/SD/NE, IL/MO/KS, GA/SC, and VA/TN showed a consistent degree of gametic phase disequilibrium coupled with uneven distribution of mating type.

TABLE 3. Analysis of molecular variance based on squared Dice-derived distances among randomly amplified polymorphic DNA haplotypes of *Setosphaeria turcica*

Source of variation	df	Sum of squares	Variance components	% Variation	<i>P</i> ^a	Fixation indices
Among regions	1	0.120	0.00085 Va	6.45	0.0215	<i>F</i> _{CT} : 0.06446
Among populations within regions	9	0.259	0.00078 Vb	5.94	<0.0001	<i>F</i> _{SC} : 0.06350
Within populations	243	2.802	0.01153 Vc	87.61	<0.0001	<i>F</i> _{ST} : 0.12387
Total	253	3.180	0.01316	...	...	...

^a Probability of having a more extreme variance component of modified *F* statistic than the observed values by chance alone. Va and *F*_{CT} are tested by permuting populations among regions. Vb and *F*_{SC} are tested by permuting haplotypes among populations, but within the same region. Vc and *F*_{ST} are tested by permuting haplotypes within populations within regions.

TABLE 4. Pairwise *F* statistics comparing state samples placed below the diagonal of the matrix^a

State	IA/SD/NE	IL/MO/KS	IN/KY	OH	MI/WI	MN	PA/NY	FL	GA/SC	NC	VA/TN
IA	0	...	...	...	...	...	...	...	...	...	...
IL	-0.0164	0	...	...	...	...	...	...	...	...	...
IN	0.0365	-0.0412	0	...	...	...	...	...	...	...	...
OH	<u>0.0878</u>	0.0432	0.0547	0	...	...	...	...	...	...	...
MI/WI	0.0239	0.0023	0.0004	-0.0067	0	...	...	...	...	...	...
MN	0.0092	-0.0194	-0.0078	0.0165	-0.0069	0	...	...	...	...	...
PA/NY	<u>0.2101</u>	<u>0.1169</u>	0.125	<u>0.2269</u>	<u>0.1207</u>	<u>0.1706</u>	0	...	...	...	...
FL	<u>0.3283</u>	<u>0.2323</u>	<u>0.1852</u>	<u>0.3111</u>	<u>0.2847</u>	<u>0.283</u>	0.0378	0	...	...	...
GA/SC	<u>0.2313</u>	<u>0.1566</u>	0.0458	0.0769	<u>0.1458</u>	<u>0.1409</u>	<u>0.1703</u>	<u>0.1941</u>	0	...	...
NC	0.0302	-0.0071	-0.0528	-0.0499	-0.0454	-0.0316	0.0182	<u>0.1622</u>	0.0549	0	...
VA/TN	<u>0.1804</u>	<u>0.1447</u>	<u>0.1329</u>	<u>0.1803</u>	<u>0.1232</u>	0.0195	0.0398	<u>0.1431</u>	0.0532	<u>0.0613</u>	0

^a Pairwise *F* statistics calculated according to Wright (50) are listed for state sample comparisons below the diagonal of the matrix. Underlined numbers indicate significant pairwise differences ($P \leq 0.05$) between states in the matrix.

This relationship suggests a clonal component to reproduction within these groups. Other state samples (IN/KY, OH, and NC) were near gametic phase equilibrium, with approximately equal proportions of mating type, consistent with sexual recombination contributing to variation. Several states (FL, PA/NY, MN, and MI/WI) showed inconsistent relationships between the index of association (near gametic phase equilibrium) and uneven distribution of mating types. These inconsistencies may be accounted for by small sample sizes, which strongly influence tests of gametic disequilibrium, or by other selective factors acting in those locations. Mating type alleles may differ in fitness in nature. Genetic data suggest possible random mating (outcrossing), but no sexual structures have been observed in the field.

Previous research has shown that tropical climates may be most suitable for sexual reproduction of *S. turcica* (3,5). This stage of the life cycle may be more likely to occur in the extreme southern United States than in typical Corn Belt states. Borchardt et al. (3,4) suggested that less recombination occurs in temperate climates, their evidence being strong gametic phase disequilibrium, uneven proportions of mating types, and lower gene and genotype diversity within temperate versus tropical populations. They propose that climatic factors in temperate zones limit the formation of fertile sexual pairings.

Severe epidemics, which are more likely under tropical conditions, may result in higher population densities and more frequent contact of sexually compatible isolates (3). Local inoculum production and dispersal are important factors in causing epidemics of NLB, and experimental evidence supports the importance of asexual reproduction in the disease cycle (29). Under conditions that are highly favorable for epidemic development, multiple generations of asexual reproduction are possible, producing clones and possibly mutants.

During years with favorable environmental conditions, susceptible hosts, or appropriate substrates for mating, genotypic diversity may increase, even in temperate climates. It is plausible that asexual conidia reach the United States via long-distance transport from Central America where the sexual stage may occur, resulting in a diverse pool of individuals that reflects sexual recombination occurring in those regions.

Genotypic diversity may increase due to sexual recombination (42). Genotypic diversities calculated for temperate populations of *S. turcica* in Europe and northern China ($H_S = 0.43$ to 0.79) (3) were lower than diversities in the United States ($H_S = 0.85$ to 1.0) (Table 5). Compared with other fungal plant pathogens recently studied, our estimates of diversity are higher than those published for *Phaeosphaeria nodorum* ($H_S = 0.82$) (28), *Rhynchosporium*

secalis ($H_S = 0.52$ to 0.92) (14), *Mycosphaerella graminicola* ($H_S = 0.57$) (27), *Pyricularia grisea* ($H_S = 0.82$ to 0.89) (8), and *Pyrenophora teres* ($H_S = 0.34$ to 0.53) (34). High levels of genotypic diversity estimated in our studies may reflect small sample sizes in some locations and the sampling of isolates over time.

The center of origin and the center of diversity of an organism may or may not exist in the same location (12,16). Genetic diversity would be expected to be high near the center of origin, in areas in which an organism has had a long history of colonization, or under conditions that are highly favorable for its growth and reproduction. At the outer limits of spread of the organism, or in environments "new" to it, the levels of diversity would be expected to be considerably lower. *S. turcica* may have its center of origin in Central America, if it is presumed that the fungus coevolved with its host, *Z. mays*. Based on studies of intercontinental genetic structure, it was proposed that another possible center of origin (or perhaps a center of diversity) could be East Africa if it coevolved with *Sorghum bicolor* (3). Borchardt et al. (3) found that Mexican isolates grouped in the center of all continental populations when plotted by multidimensional scaling, and found the highest number of polymorphic bands in this location. However, they were unable to resolve the question of origin, because gene diversity in Mexico was not significantly higher than that in Embu, Kenya. The proximity of the eastern United States to Central America, the potential site of origin for *S. turcica*, may account for high levels of genotypic diversity found in our studies compared with that of populations from temperate zones in Europe and China.

A small sample of isolates infective to *Sorghum bicolor* and *Sorghum halepense* from NC and IN was included in our analysis to examine the relationship of these haplotypes to those from maize-infecting isolates. *Sorghum*-infecting isolates were not all of identical haplotype. No haplotypes were shared by maize-infecting and sorghum-infecting isolates. Isolates having different host specificity may have coexisted in the United States for a longer time period than those sampled in Europe (3).

Presence of several haplotypes over long distances provides direct evidence of migration of *S. turcica* within the eastern United States. Barriers to the movement of conidia are not in evidence as was the situation in Europe, where populations were separated by the Alps (4) or by continental isolation (3). Our studies did not reveal clear separation of multilocus haplotypes by state of origin. Shared haplotypes between different states indicated substantial gene flow among the states sampled.

In the eastern United States, overrepresentation of haplotypes was not as pronounced as the European case (4). The highest

TABLE 5. Genotypic diversity (Shannon index, H_S), gene diversity (Nei's average gene diversity, H_N), significance of recombination (Brown's index of multilocus association, I_A), and mating type frequencies in state samples of *Setosphaeria turcica*

Sample location	Genotypic diversity ^a			Gene diversity		Multilocus allele association ^b			Mating type ^c	
	<i>N</i>	<i>M</i>	H_S^d	b^e	H_N^d	I_A	V_O	<i>L</i>	<i>MAT1-1</i>	<i>MAT1-2</i>
IA/SD/NE	44	32	0.89	16	0.167	0.557	3.04 ≤	3.09 ^f	9**	35**
IL/MO/KS	58	41	0.85	19	0.197	0.478	3.63 =	3.62 ^f	23	35
IN/KY	12	12	1.00	11	0.192	0.102	2.58 <	3.67 ^f	8	4
OH	14	14	1.00	12	0.171	0.046	2.31 <	3.44 ^f	7	7
MI/WI	15	15	1.00	14	0.183	0.412	3.63 <	4.12 ^f	4*	11*
MN	25	21	0.91	13	0.183	0.144	2.69 <	3.73 ^f	7*	18*
PA/NY	12	11	0.95	10	0.153	0.07	2.15 <	3.25 ^f	1**	11**
FL	18	16	0.95	13	0.196	0.02	2.62 <	3.95 ^f	15**	3**
GA/SC	13	13	1.00	16	0.259	1.441	8.38 >>	5.22	10*	3*
NC	24	23	0.98	19	0.238	0.387	4.04 <	5.06 ^f	11	12
VA/TN	16	16	1.00	19	0.239	0.757	6.26 >>	5.21	2**	14**

^a *N* = sample size and *M* = number of multilocus randomly amplified polymorphic DNA haplotypes per sample.

^b I_A = Brown's index of multilocus association, V_O = observed variance (test statistic), and *L* = lower confidence limit.

^c Number of isolates of each mating type in state samples. * and ** = H_O (equal distribution of mating types) rejected at $\alpha = 0.05$ and 0.01 , respectively.

^d None of the H_S or H_N values were significantly different from one another at $\alpha = 0.05$, according to a simple *t* test.

^e Number of polymorphic bands of the 21 possible.

^f $H_O: I_A = 0$ at $\alpha = 0.05$.

representation of a single haplotype in American isolates was only 6%. In European samples, nearly one-third of the isolates were of the predominant haplotype, others likely arising via mutation (3). Some European haplotypes could not be explained by mutation alone, and it was hypothesized that sexual recombination played a role in temperate climates, even though the sexual stage has not been found in the field. Overall, the genotypic diversities were higher than our expectation for highly clonal populations. This effect may be attributable to sexual reproduction, small sample size, or collection of isolates over time.

Our expectation of lower gene diversity in samples from the northern-most states in the United States, which are farther removed from the probable center of origin, was not supported statistically. Though there were no significant differences among state samples, the highest gene diversities were found in the southeastern states, NC, GA/SC, and VA/TN, with the exception of FL, which may have been the result of small sample size. Lowest gene diversity was found in PA/NY, which is at a greater geographic distance from the likely source of the pathogen than the midwestern states. The lack of significant differences in average gene diversity among state populations indicates that no real barriers delineate populations of *S. turcica* in the eastern United States.

Analysis of molecular variance showed that regional variation and variation among states within regions accounted for only a small portion of that seen in these samples. Most variation was concentrated within state groups. Sampling over time may account for some of the variation seen, as would grouping isolates within a state representing multiple locations. However, state samples differed in RAPD band frequencies, evident from multidimensional scaling plots based on average taxonomic distance among states and reinforced by state pairwise F_{ST} calculations. The largest differences in marker frequencies occurred among FL, PA/NY, VA/TN, and GA/SC. These groups were distant both from one another and from a larger grouping of states showing greater similarity. The smallest distances lay between IL/MO/KS, OH, IN/KY, IA/SD/NE, MN, MI/WI, and NC. Midwestern states in the Corn Belt share similar cropping strategies, hybrids deployed, and climatic conditions during the growing season.

It is somewhat surprising that such small genetic distances were found between NC and the midwestern samples given the geographic distances involved. There are few natural geographic barriers to long-distance transport of inoculum from earlier planted fields in NC to midwestern cornfields. Similarity of hybrids recommended for use in these areas may also account for genetic similarity of pathogen samples. For example, commercial corn seed producer Pioneer Hi-Bred International uses a hybrid maturity (CRM) range for SC, NC, and VA of 87 to 132 days versus IL with a CRM of 101 to 119 days. Of the hybrids recommended for SC, NC, and VA, 46% of them also were recommended for the state of IL.

All four major races of *S. turcica* were present in the U.S. collections studied. Shared similarities among states based on RAPD markers were also reflected in representation of races in different states. Among the survey isolates, only races 0 and 1 were present in FL, PA/NY, and OH. Race 23 was found in NC, IL/MO/KS, IA/SD/NE, and MI/WI. This race occurred at the lowest frequencies overall in the eastern United States. Among isolates from VA collected in 1957, 23 and 23N isolates were found. Races 23 and 23N were not officially reported until the 1980s, but evidently were present in the United States at low frequencies much earlier, even though *Ht2*, *Ht3*, and *HtN* were not deployed on a commercial scale. In most states, race 0 was predominant in the absence of strong selection for virulence to *Ht* genes. In three state groups, NC, VA/TN, and PA/NY, race 1 isolates made up the highest proportion of isolates. This emphasizes that even in populations where migration is common and widespread, different selective forces shape populations at the local level.

More variability was evident in samples from the eastern United States than anticipated based on other studies of temperate populations of *S. turcica*. Long-distance migration is likely within the states studied and contributes to genetic diversity, supported by the lack of subdivision on a regional or state level. High genotypic diversities in some states may be the result of sexual recombination, migration, or sampling effects. However, unequal proportions of mating types in seven of the states analyzed provide evidence of the importance of asexual reproduction in this pathosystem.

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