Identification and Genetic Diversity of Formae Speciales of *Setosphaeria turcica* in China

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Abstract

Tang, L., Gao, Z. G., Yao, Y., and Liu, X. 2015. Identification and genetic diversity of formae speciales of *Setosphaeria turcica* in China. Plant Dis. 99: 482-487.

Setosphaeria turcica is an important pathogen that causes northern leaf blight of corn and sorghum. In total, 188 isolates were isolated from diseased leaves of maize and sorghum in China. However, differences in morphology were not apparent among *S. turcica* isolates derived from different hosts. The formae speciales were identified as *S. turcica* f. sp. *zeae* and *S. turcica* f. sp. *sorghii*. Universally primed polymerase chain reaction (UP-PCR) analysis led to the conclusion that genetic differences were

present among these formae speciales and divided these isolates into five clades. The isolates of each clade belonged to the same forma specialis, and different formae speciales were assigned to different clades. The results of the present study demonstrated that pathogenic specialization of the *S. turcica* isolate correlated more closely with genetic diversity than geographical distribution. Therefore, combined with assays for pathogenicity, UP-PCR analysis yields an enhanced understanding of the formae speciales of *S. turcica*.

Northern leaf blight of corn and sorghum caused by *Setosphaeria turcica* (Luttr.) K. J. Leonard & Suggs (21) is one of the important foliar diseases of corn and sorghum wherever they are cultivated (13). Historically, isolates of *S. turcica* were divided into formae speciales according to their virulence for a particular host or group of hosts. Since Mitra (26) first reported specialization of *S. turcica* on maize or sorghum, Johnson grass and Sudan grass were identified as hosts as well (3,4,20,21,25,29,30). Therefore, *S. turcica* is classified into the following types: *S. turcica* f. sp. *zeae*, *S. turcica* f. sp. *sorghii*, and *S. turcica* f. sp. *complexa*. *S. turcica* f. sp. *zeae* is only virulent on maize, *S. turcica* f. sp. *sorghii* infects sorghum and Sudan grass, and *S. turcica* f. sp. *complexa* infects each of the hosts listed here. Therefore, two formae speciales of *S. turcica*, *S. turcica* f. sp. *zeae* and *S. turcica* f. sp. *sorghii*, only infect specific compatible hosts and induce typical spots. *S. turcica* f. sp. *complexa* also is called a nonforma specialis (1).

Previous research on the formae speciales of *S. turcica* was limited to pathogenicity tests of host-pathogen interactions; however, to our knowledge, there are no studies of the molecular mechanism and genetic basis of pathogenic specialization of *S. turcica* in China. Moreover, determining pathogenicity by analyzing host-pathogen interactions is subjective and inaccurate and, therefore, not comprehensive. For example, molecular biology techniques are widely used to explore genetic variability of fungi (10,15,36-38). Therefore, analyzing the genetic diversity of pathogens represents an important aspect of the comprehensive analysis of formae speciales (37).

To our knowledge, there are no published studies of the genetic relationships among formae speciales of *S. turcica*. Universally primed polymerase chain reaction (UP-PCR) is a fingerprinting method that amplifies the DNA of any organism without previous knowledge of DNA sequences and generates multiband profiles (fingerprints) using gel electrophoresis (7-9,23,28). UP-PCR primers can be designed to amplify the intergenic variable regions of genomes (6). For example, the UP-PCR technique was applied successfully to characterize the genomes of pathogens such as *Fusarium* spp., *Rhizoctonia solani*, *Trichoderma* spp., *Gliocladium* spp., and *Pyrenophora graminea* (7,15,23).

The goals of this study were to determine the formae speciales of *S. turcica* in China and to analyze the relationships among genetic diversity, geographic distribution, and pathogenic specialization.

Materials and Methods

Purification and cultivation of pathogenic isolates. The isolates studied here were obtained using a tissue separation method (14) from samples of infected leaves of maize and sorghum collected from various regions of China. Monosporic cultures of *S. turcica* isolates were cultivated on potato dextrose agar (PDA; 200 g of potato, 20 g of glucose, 18 g of agar, and 1 liter of water) at 25°C.

DNA extraction and identification of internal transcribed spacer regions. Each isolate was cultivated for 7 days on potato dextrose broth (PDB; 200 g of potato extract, 20 g of glucose, and 1 liter of water) at 25°C. The mycelia in PDB were filtered with the double layer of sterile gauze, washed with sterile distilled water, and dried using FreeZone 2.5-liter Freeze Dry Systems (Light Ace HK TLD, Beijing) until they were completely dry (38). The dried mycelia were placed in liquid nitrogen in a mortar and ground with a pestle into a fine powder. Total DNA was extracted using an Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). DNA was quantified by measuring optical density at 260 or 280 nm (33) and using 1.2% agarose gel electrophoresis. Experimental isolates were identified by comparing their internal transcribed spacer (ITS) sequence with that of an *S. turcica* reference isolate (National Center for Biotechnology Information [NCBI] accession number KF278460.1) maintained in our laboratory. ITS reactions (50 µl) contained 5 µl of 10× buffer, 3 µl of 25 mM MgCl₂, 4 µl of 2.5 mM dNTP, 1 µl of 10 mM ITS1 primer (16,17), 1 µl of 10 mM ITS4 primer (16,17), 0.8 µl of Taq polymerase (Tiangen Biotech, Beijing) at 2.5 U µl⁻¹, 1 µl of 100 ng of DNA, and 34.2 µl of double-distilled (dd)H₂O. The PCR program was as follows: 5 min at 94°C; followed by 35 cycles at 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min, with final extension at 72°C for 10 min (38). The amplified fragments were separated through a 1.5% agarose gel electrophoresis containing 1× Tris-borate-EDTA (TBE). The 1.5% agarose gel containing Goldview (Tiangen Biotech), which is a new and safe nucleic acid dye compared with ethidium bromide, was prepared 30 min before electrophoresis for 40 min at 110 V. The gel was visualized to confirm the reproducibility of the band patterns and photographed using a UV Transilluminator (UVItec, Cambridge, UK). Nucleotide sequencing was performed by Sangon Biotech.

Pathogenicity tests. Susceptible cultivars were as follows: 'Huangzaosi' maize (lacking a resistance gene), 'LR115' sorghum, and 'Yuantai023'

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Accepted for publication 19 November 2014.

<http://dx.doi.org/10.1094/PDIS-06-14-0570-RE>
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Table 1. *Setosphaeria turcica* isolates used for universally primed polymerase chain reaction and their accession numbers

Strains	Accession numbers
CCG1326	KJ922714.1
SHG1303	KJ922737.1
PLG1306	KJ922734.1
JPG1302	KJ922726.1
SYG1306	KJ922739.1
YYG1302	KJ922750.1
ZMDG1308	KJ922752.1
YCG1301	KJ922748.1
LLG1302	KJ922728.1
XZG1312	KJ922746.1
CZG1304	KJ922718.1
HSG1302	KJ922723.1
TLG1303	KJ922741.1
LPG1304	KJ922730.1
XAG1303	KJ922744.1
MHKG1309	KJ922732.1
DQG1301	KJ922721.1
SYG1303	KJ922740.1
JPY1302	KJ922727.1
DLY1308	KJ922720.1
DQY1306	KJ922722.1
SHY1309	KJ922738.1
CCY1303	KJ922715.1
MHKY1302	KJ922733.1
PLY1305	KJ922735.1
YCY1324	KJ922749.1
YYY1319	KJ922751.1
ZMDY1311	KJ922753.1
LLY1305	KJ922729.1
XZY1312	KJ922747.1
CZY1321	KJ922719.1
HSY1309	KJ922724.1
TLY1308	KJ922742.1
LPY1313	KJ922731.1
XAY1302	KJ922745.1
JNY1315	KJ922725.1
QDY1307	KJ922736.1
CDY1316	KJ922716.1
CQY1310	KJ922717.1
WQY1308	KJ922743.1

Sudan grass. The plants were grown in a greenhouse in plastic pots (three plants per pot) to the six-leaf stage before inoculation. Conidia were washed from 10-day-old pure cultures grown on PDA at 25°C using sterile distilled water. Conidial suspensions (1×10^6 conidial/ml) were prepared using sterile distilled water. Tween-20 was added into the suspension to a final concentration of 0.02%. Each conidial suspension was used to inoculate maize, sorghum, and Sudan grass at the same time. To keep the leaf blades wet, plants were incubated at 20 to 26°C for 48 h in a plastic tent. The experimental design was completely randomized using three biological replicates. The plants were examined for symptoms of disease after 15 days and compared with those of corn northern leaf blight (Centro Internacional de Mejoramiento de Maíz y Trigo), which served as a positive control.

UP-PCR analysis. In all, 23 isolates of *S. turcica* f. sp. *zeae* and 17 of *S. turcica* f. sp. *sorghii* were selected as representatives of each forma specialis (Table 1). The UP primers AA2M2, AS15inv, AS4, AS15, HE45, L45, L15, L15/AS19, L21, 0.3-1, 0.3-2, 3-1, and 3-2 (32) were used to amplify the total DNA of each. PCR reactions were performed in a final volume of 20 μ l and contained 2 μ l of 10 $\times$ buffer, 2 μ l of 25 mM $MgCl_2$, 1.6 μ l of 2.5 mM dNTP, 1 μ l of 10 mM UP primer, 0.6 μ l of Taq polymerase (Tiangen Biotech) at 2.5 U μ l⁻¹, 1 μ l of 20 ng of DNA, and 11.8 μ l of ddH₂O. The thermocycle programs was as follows: denaturation for 2 min at 94°C, 40 s at 53°C, and 30 s at 72°C; followed by 30 cycles at 92°C for 20 s, 53°C for 40 s, and 72°C for 30 s; and final extension at 72°C for 2 min (18). PCR-amplified fragments were separated on a 1.5% agarose gel electrophoresis containing 1 $\times$ TBE. The 1.5% agarose gel containing Goldview (Sangon Biotech) was prepared 30 min before electrophoresis for 40 min at 110 V. The gel was visualized to confirm the reproducibility of the bands patterns and photographed using a UV transilluminator (UVItect).

Data analysis. Amplified bands generated using each primer set were assigned as present = 1 or absent = 0, followed by cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) (31) implemented in the NTSYS-pc program, version 2.02 (32).

Results

Morphological studies and identification of ITS sequences. Colony morphology, hyphae, conidiophores, and conidia of 188 isolates of *S. turcica* collected in China were examined in the study. Colonies were ash black and circular with gray aerial hyphae. Brown

Fig. 1. Morphology of *Setosphaeria turcica* from maize (isolate SYY1326): **A**, colony morphology; **B**, hyphae and conidiophores morphology; **C**, conidial morphology. Morphology of *S. turcica* from sorghum (strain JPG1306): **D**, colony morphology; **E**, hyphae morphology; **F**, conidial morphology. Bars: **B** and **E** = 20 μ m, **C** and **F** = 10 μ m.

filiform conidiophores were erect or flexuous, single or tufted, with larger basal cells, light brown tips, and two to six cross septa, and they were 32.6 to 180 μm long and 5.6 to 13.1 μm wide. Conidia were olive-brown, long, and spindle-shaped, with long elliptical apical cells, pointed cone basal cells, and obvious umbilic points outside

the basal cell with two to eight cross septa, and they were 48.7 to 126.9 μm long and 11.2 to 23.6 μm wide. The morphology, colony size, hyphae, conidiophores, and conidia were similar among all isolates (Fig. 1). There were no differences in morphology among *S. turcica* isolates derived from different hosts. There was as much as 99%

Fig. 2. Pathogenicity tests of *Setosphaeria turcica* on maize and sorghum. **A**, Typical disease spot of *S. turcica* f. sp. *zeae* on maize; **B**, no visible reaction of *S. turcica* f. sp. *zeae* on sorghum except some flecks; **C**, no visible reaction of *S. turcica* f. sp. *zeae* on Sudan grass except some flecks; **D**, typical disease spot of *S. turcica* f. sp. *sorghi* on sorghum; **E**, typical disease symptom of *S. turcica* f. sp. *sorghi* on Sudan grass; **F**, no visible reaction of *S. turcica* f. sp. *sorghi* on maize.

Table 2. Isolates of *Setosphaeria turcica* included in the study, their formae specialis, and origins

Formae specialis, strains ^a	Numbers	Geographic origin ^b
<i>S. turcica</i> f. sp. <i>sorghi</i>		
PLG1301, PLG1302, PLG1303, PLG1304, PLG1305, PLG1306*	6	Pingliang, Gansu
DQG1301*, DQG1302, DQG1303	3	Daqing, Heilongjiang
HGG1301, HGG1302	2	Hegang, Heilongjiang
SHG1303*, SHG1311	2	Suihua, Heilongjiang
JPG1302*, JPG1304, JPG1305, JPG1306	4	Jianping, Liaoning
FCG1303, FCG1305, FCG1307	3	Fengcheng, Liaoning
SYG1302, SYG1303, SYG1305, SYG1306*, SYG1307, SYG1310, SYG1311, SYG1312, SYG1317	9	Shenyang, Liaoning
LLG1302*, LLG1321, LLG1323	3	Lvliang, Shanxi
XZG1308, XZG1312*	2	Xinzhou, Shanxi
CZG1304*, CZG1307, CZG1318	3	Cangzhou, Hebei
HSG1301, HSG1302*	2	Hengshui, Hebei
TLG1302, TLG1303*, TLG1306	3	Tongliao, Neimeng
LPG1301, LPG1302, LPG1303, LPG1304*, LPG1305	5	Luoping, Yunnan
XAG1301, XAG1302, XAG1303*, XAG1304, XAG1305	5	Xi'an, Shaanxi
YLG1301, YLG1302, YLG1307	3	Yangling, Shaanxi
YCG1301*, YCG1302, YCG1305	3	Yichuan, Henan
LYG1301, LYG1303, LYG1306, LYG1309	4	Luoyang, Henan
ZMDG1308*, ZMDG1311, ZMDG1322	3	Zumadian, Henan
YYG1302*, YYG1304, YYG1305	3	Yuanyang, Henan
MHKG1302, MHKG1304, MHKG1309*, MHKG1314	4	Meihekou, Jilin
CCG1312, CCG1317, CCG1326*	3	Changchun, Jilin
<i>S. turcica</i> f. sp. <i>zeae</i>		
SYG1301, SYG1303*, SYG1306, SYG1326	4	Shenyang, Liaoning
TLY1302, TLY1305, TLY1311	3	Tieling, Liaoning
JPY1301, JPY1302*, JPY1307	3	Jianping, Liaoning
DLY1308*, DLY1316	2	Dalian, Liaoning
DDY1321	1	Dandong, Liaoning

(continued in next column)

^a Isolates marked with an asterisk were used in universally primed polymerase chain reaction.

^b Region and province in China.

Table 2. (continued from preceding column)

Formae specialis, strains ^a	Numbers	Geographic origin ^b
WFDY1314, WFDY1332	2	Wangfangdian, Liaoning
FCY1303, FCY1304, FCY1309, FCY1311	4	Fengcheng, Liaoning
HEBY1304, HEBY1307, HEBY1310, HEBY1311	4	Habin, Heilongjiang
DQY1301, DQY1305, DQY1306*, DQY1309, DQY1321	5	Daqing, Heilongjiang
SHY1303, SHY1309*, SHY1313	3	Suihua, Heilongjiang
HGY1308, HGY1311, HGY1313, HGY1315	4	Hegang, Heilongjiang
CCY1302, CCY1303*, CCY1309	3	Changchun, Jilin
MHKY1301, MHKY1302*, MHKY1311, MHKY1320, MHKY1321	5	Meihekou, Jilin
SPY1302, SPY1303, SPY1305, SPY1306	4	Siping, Jilin
PLY1301, PLY1302, PLY1305*	3	Pingliang, Gansu
LZY1302, LZY1304, LZY1305, LZY1312	4	Lanzhou, Gansu
QDY1305, QDY1307*, QDY1309	3	Qingdao, Shandong
JNY1314, JNY1315*, JNY1318	3	Jinan, Shandong
CDY1314, CDY1315, CDY1316*	3	Chengdu, Sichuan
CQY1305, CQY1307, CQY1309, CQY1310*	4	Congqing
LYY1301, LYY1303, LYY1304, LYY1306	4	Luoyang, Henan
YCY1305, YCY1312, YCY1324*	3	Yichuan, Henan
YYY1314, YYY1315, YYY1319*	3	Yuanyang, Henan
ZMDY1308, ZMDY1311*, ZMDY1319	3	Zhumadian, Henan
TTY1302, TTY1307, TTY1311	3	Taiyuan, Shanxi
LLY1303, LLY1305*	2	Lvliang, Shanxi
XZY1311, XZY1312*, XZY1315	3	Xinzhou, Shanxi
CZY1301, CZY1313, CZY1321*	3	Cangzhou, Hebei
HSY1304, HSY1307, HSY1309*	3	Hengshui, Hebei
TLY1306, TLY1308*, TLY1309, TLY1310, TLY1312, TLY1314	6	Tongliao, Neimeng
LPY1307, LPY1309, LPY1313*	3	Luoping, Yunnan
XAY1301, XAY1302*, XAY1303, XAY1309	4	Xi'an, Shaanxi
YLY1306, YLY1310, YLY1316	3	Yangling, Shaanxi
WQY1308*, WQY1310, WQY1322	3	Wuqing, Tianjin
Sum	188	...

Fig. 3. Geographic distributions of the formae specialis of *Setosphaeria turcica*. Red areas are common geographic distributions of *S. turcica* f. sp. *sorghi* and *S. turcica* f. sp. *zeae*, and green areas are geographic distributions of *S. turcica* f. sp. *zeae* but no *S. turcica* f. sp. *sorghi*.

Table 3. Primers, their sequence and number of bands scored in universally primed polymerase chain reaction UP-PCR analysis of *Setosphaeria turcica*.

Primers	Nucleotide sequence 5'–3'	Scored bands	Polymorphic bands	Homology ratio (%)	Polymorphism Ratio (%)
L21	GGATCCGAGGGTGGCGTTCT	13	10	...	...
HE45	GTAAAACGAGGCCAGT	9	4	...	...
0.3-2	TGAGGACAACGGTTCT	10	10	...	...
AA2M2	CTGCGACCCAGAGCGG	14	12	...	...
AS15inv	CATTGCTGGCGAATCGG	16	15	...	...
Total	...	62	51	17.7	82.3

Fig. 4. Unweighted pair group method with arithmetic mean dendrogram of analyzed *Setosphaeria turcica* f. sp. *zeae* and *S. turcica* f. sp. *sorghi* isolates obtained from coefficient similarity matrix using universally primed polymerase chain reaction marker data.

DNA sequence identity (the value of “sort by ident”) compared with the reference isolate (NCBI accession number KF278460.1) among all isolates. Taken together, the results of these analyses indicate that all isolates were *S. turcica*.

Pathogenicity analysis. All 188 isolates revealed significant differences in virulence compared with the corn northern leaf blight control. Thus, maize blades were covered with fusiform necrotic spots after inoculation with isolates from maize, which had dark-brown centers and light-gray margins. The incompatible reactions observed on sorghum and Sudan grass were fuchsia-colored flecks (Fig. 2A to C). The long fusiform necrotic spots with light-brown centers and fuchsia-colored margins were formed on the blades of the sorghum and Sudan grass after inoculation with isolates from sorghum. Incompatible reactions appeared symptomless on maize (Fig. 2D and E). There were 113 *S. turcica* f. sp. *zeae* and 75 *S. turcica* f. sp. *sorghii* isolates among the 188 isolates (Table 2) but no isolates of *S. turcica* f. sp. *complexa*.

Two formae speciales of *S. turcica* were widely dispersed among the regions where maize and sorghum are cultivated in China (Fig. 3; Table 2). *S. turcica* f. sp. *zeae* and *S. turcica* f. sp. *sorghii* were present in 34 regions of 14 provinces and in 21 regions of 10 provinces, respectively. Because sorghum is cultivated in smaller areas compared with maize, *S. turcica* f. sp. *sorghii* was not isolated from plants in certain regions such as Shandong province (Qingdao and Jinan), Sichuan province (Chengdu), Chongqing, Tianjin (Wuqing), Gansu province (Lanzhou), Liaoning province (Dalian, Tieling, Dandong, Wangfangdian), Jilin province (Siping), Heilongjiang province (Habin), and Shanxi province (Taiyuan). Therefore, pathogenicity did not directly correlate with the geographic distributions in all isolates.

UP-PCR analysis. In all, 17 *S. turcica* f. sp. *sorghii* isolates and 23 *S. turcica* f. sp. *zeae* isolates were analyzed using UP-PCR. Primers L21, HE45, 0.3-2, AA2M2, and AS15inv generated polymorphic banding patterns and were used for UP-PCR analysis (Table 3). There were 62 amplified bands and 51 polymorphic bands. The homology to polymorphism ratios in the bands from the five UP-PCR primers was 17.7 and 82.3%, respectively.

A dendrogram generated from the UPGMA analysis is shown in Figure 4. The phenon line, representing the mean between similar isolates, was drawn at 78%. At this level, the 40 *S. turcica* isolates were divided into five clades. Clades 1 and 3 included *S. turcica* f. sp. *zeae*. Clades 2, 4, and 5 included *S. turcica* f. sp. *sorghii*. The isolates of each clade belonged to the same forma specialis, and different formae speciales were assigned to different clades. Consistent with the results of pathogenicity tests and findings that two formae speciales were widespread in the planting areas of maize and sorghum in China, the UP-PCR analysis indicates a correlation between pathogenic specialization and genetic diversity. Therefore, UP-PCR is useful for the study of genetic relations among the formae speciales of *S. turcica* and reveals a high level of genetic diversity among these pathogens.

According to their geographic distribution, the same forma specialis from different provinces were present in the same clade. For example, LLY1305, CCY1303, and SHY1309 were assigned to clade 3, although they were isolated from the provinces of Shanxi, Jilin, and Helongjiang, respectively. However, in some instances, the same forma specialis from the same province was classified in a different clade. For example, DQG1301 and SHG1303 were assigned to in clades 2 and 5, respectively, although they were isolated from Helongjiang province. Therefore, UP-PCR analysis did not indicate a correlation between and genetic diversity and geographic distribution.

Discussion

No differences in morphology were observed among the *S. turcica* strains derived from different hosts, which is consistent with previous reports (1,3,4,13,20,21,25,26,29,30,34). The pathogenicity assays performed in the present study identified 113 *S. turcica* f. sp. *zeae* isolates and 75 *S. turcica* f. sp. *sorghii* isolates but did not identify the *S. turcica* f. sp. *complexa* among a collection of 188 isolates. Two formae speciales of *S. turcica* were widespread in regions in China where maize and sorghum are cultivated.

UP-PCR analysis demonstrated genetic differences among the formae speciales of *S. turcica* and divided them into five clades that include the same forma specialis and isolates of from the different provinces in the same clade and the same forma specialis of the same provinces in different clades. The results of pathogenicity assays and UP-PCR analysis indicated a closer correlation between pathogenic specialization and genetic diversity but not geographic distribution. These conclusions are consistent with those of other studies (5,27,37).

Pathogenicity tests are influenced by variables such as temperature (35), age of the host (12), and the method of inoculation (19). Therefore, the goal of the present study was to develop a PCR-based method for studying genetic diversity of formae speciales of *S. turcica* isolates to better understand disease control, population dynamics, and epidemiology. The UP-PCR molecular marker was designed to identify genomic differences without previous knowledge of the target DNA sequences. These results were analyzed for their correlation with colony morphology (22), pathogenicity, geographic distribution (27), and formae speciales (2,11), as well as other factors (24). Therefore, the present study illustrates the utility of UP-PCR technology for supplementing pathogenicity tests to accurately characterize the genetic diversity and phylogenetic relationships among the formae speciales of *S. turcica*. However, because of the limited number of geographic regions studied here and the insufficient number of *S. turcica* f. sp. *complexa* isolates, further research is required fully to characterize the formae speciales in *S. turcica*.

Acknowledgments

We thank X. Xu and Y. Jiang (Institute of Plant Protection, Liaoning Academy of Agricultural Sciences, Shenyang, China) for providing the *S. turcica* stains. This project was supported by the National Key Technology Research and Development Program of Program Program (number 2012BAD19B04) and Research Projects in Liaoning Province (number 2014201003).

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