Genetic Diversity and Identification of Wilt and Root Rot Pathogens of Tomato in China

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Abstract

Fungal wilt and root rot diseases affecting tomato have become prevalent in China in recent years and have caused considerable damage. In 2016 to 2018, symptoms of putative wilt and root rot diseases were observed in several locations in tomato cultivars with resistance to *Fusarium oxysporum* f. sp. *lycopersici* races 1 and 2. The objective of this study was to identify the causative agents of wilt and root rot of tomato in China and provide a basis for disease prevention and resistance breeding programs. Based on DNA sequence analyses of the internal transcribed spacer (ITS) region, 91 isolates from the roots of tomato plants showing symptoms of wilt and root rot were identified, including *F. oxysporum* (64 isolates), *Fusarium solani* (11 isolates), *Fusarium proliferatum* (2 isolates), *Fusarium graminearum* (2 isolates), *Fusarium equiseti* (1 isolate), *Pythium aphanidermatum* (6 isolates), *Ascomycota* sp. (2 isolates), and *Plectosphaerella cucumerina* (3 isolates). *F. oxysporum* accounted for 70.33% of the isolates obtained. In this case, using PCR-based

methods for differentiation of *F. oxysporum*, we identified several *formae speciales* and races of *F. oxysporum*: 7 isolates were identified as *F. oxysporum* f. sp. *lycopersici* race 1, 2 isolates as *F. oxysporum* f. sp. *lycopersici* race 2, 35 isolates as *F. oxysporum* f. sp. *lycopersici* race 3, and 13 isolates as *F. oxysporum* f. sp. *radicis-lycopersici*. Pathogenicity tests revealed 55 isolates of tomato wilt and root rot pathogens to be virulent. This study demonstrated that *F. oxysporum* f. sp. *lycopersici* race 3 was the most widespread and highly virulent race among these tomato pathogens in China, followed by *F. oxysporum* f. sp. *radicis-lycopersici*. Therefore, the development of resistant varieties of tomato against *F. oxysporum* f. sp. *lycopersici* race 3 and *F. oxysporum* f. sp. *radicis-lycopersici* would aid efforts to develop effective disease management strategies.

Keywords: fungi, pathogen detection, vegetables

Tomato (*Solanum lycopersicum*) is a very important vegetable crop in China, with approximately 59.6 million tons of tomatoes harvested from 1 million ha in 2017 (FAOSTAT 2017). Disease surveys in 2016 to 2018 indicated that tomato was severely affected by wilt and root rot complexes, which caused severe damage in commercial tomato production fields, leading to substantial reductions in yield. Timely and appropriate disease management strategies to counter this damage require rapid and reliable detection and identification of the species and race of potential pathogens.

Accurate identification of the causal organisms is essential because tomato is affected by >200 diseases (Lukyanenko 1991); of these, fungal wilt and root rot diseases are the most important and destructive diseases worldwide. Various pathogens associated with tomato wilt and root rot have been reported, including *Phytophthora* (Lievens et al. 2004), *Pythium* spp. (Callaghan et al. 2019), *Pseudomonas syringae* (Hyder et al. 2019), and *Fusarium oxysporum* (Hirano and Arie 2006). Of these, *F. oxysporum* is a phylogenetically diverse species and distributed worldwide. It is a well-known producer of mycotoxin, and its consequent destructive potential makes its identification and management a high priority (Irzykowska et al. 2012). Virulent isolates of *F. oxysporum* are distinguished as *formae speciales* on the basis of their host specificity, and >150 *formae speciales* have been identified (Armstrong and Armstrong 1981;

Lievens et al. 2008, 2012). The two important *formae speciales* of *F. oxysporum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici*, display symptomatological, genetic, and epidemiological differences and have been extensively studied (Baysal et al. 2009; Çolak and Biçici 2013; Manzo et al. 2016; Nirmaladevi and Srinivas 2012). Physiological races of *F. oxysporum* are classified according to their ability to infect plants and cause disease in different cultivars containing different resistance loci (Nirmaladevi and Srinivas 2012). Three races (1, 2, and 3) of *F. oxysporum* f. sp. *lycopersici* have been distinguished (Alexander and Tucker 1945; Grattidge and O'Brien 1982; Mes et al. 1999). Traditionally, identification has relied on morphological and physiological factors. However, the *formae speciales* and races of *F. oxysporum* are morphologically very similar and are thus very difficult to distinguish.

Recently, DNA-based molecular markers have been broadly used to identify various species, *formae speciales*, and physiological races of *F. oxysporum*. Different markers such as internal transcribed spacer (ITS) regions, translation elongation factor-1 α (TEF-1 α), intergenic spacer (IGS) ribosomal DNA (rDNA), mating type genes (MAT1-1 and MAT1-2), exopolysaccharuronase gene (*pgx4*), and endopolysaccharuronase gene (*pg1*) have been reported for the identification of isolates affecting tomato. ITS sequences have been generally used for phylogenetic analysis in fungi (Bao et al. 2002; Sampietro et al. 2010; Schoch et al. 2012). Likewise, TEF-1 α , *pgx4*, MAT1-1, and MAT1-2 genes have been used to elucidate evolutionary relationships between *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* isolates (Lievens et al. 2009). Hirano and Arie (2006) compared the partial nucleotide sequences of *pg1* and *pgx4* genes from isolates of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* from Japan and established a PCR-based method to differentiate the virulent types of *F. oxysporum* in tomato. Do Amaral et al. (2013) used random amplification of polymorphic DNA, inter-simple sequence repeats, and restriction fragment length polymorphism–intergenic spacer analysis for identifying races 1, 2, and 3 of *F. oxysporum* f. sp. *lycopersici*. Si Mohammed et al. (2016) proposed the classification and identification of *F. oxysporum* isolates from tomato in Algeria on the basis of the TEF-1 α gene.

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The main objective of this study was to identify the causative agents of wilt and root rot of tomato in China so as to inform disease management and resistance breeding programs. Isolates from the roots of tomato plants showing symptoms of wilt and root rot were identified via DNA sequence analyses of ITS regions and via the *pgx4* gene and molecular method reported by Hirano and Arie (2006). The nucleotide sequences of ITS and *pgx4* were obtained, and phylogenetic analyses of different isolates were performed. In addition, to confirm the predominant virulent isolates that were causing tomato wilt and root rot diseases, we compared the virulence of the isolates in the tomato variety ‘T15-196’ (genes I and I-2 present), which is resistant to *F. oxysporum* f. sp. *lycopersici* races 1 and 2.

Materials and Methods

Sampling and fungal isolates. Samples used for isolation of potential fungal pathogens were collected from 2016 to 2018 at the following high-incidence locations and provinces: Xiaoshan, Haining, and Jinhua (Zhejiang); Sanya (Hainan); Xi’an (Shanxi); and Zibo and Shouguang (Shandong). The 91 isolates used in this study were isolated from the roots of tomato plants with symptoms of leaf yellowing, wilting, root rot, crown rot, or death. Affected roots of the collected plant samples were washed under running tap water to remove soil particles. The roots were cut into 0.3 to 0.6-cm pieces. The cut pieces were surface sterilized with 70% ethanol for 40 to 50 s, rinsed with sterile distilled water, blotted dry on sterile filter paper, and placed on potato dextrose agar (PDA) medium in 6-cm-diameter plastic Petri dishes. The dishes were incubated in a growth chamber at 25°C. After 2 days, subculturing was performed to obtain pure cultures of the isolates. The pure cultures were purified by the single spore isolation technique, and the purified isolates were incubated on PDA medium for 7 to 10 days at 25°C, with long-term storage at 4°C.

Inoculum preparation. Each isolate was inoculated into 100 ml sterilized liquid PDA medium for 48 h and shaken at 180 rpm, 25°C. The spore suspensions thus obtained were measured in a cell counting chamber and adjusted to 1 to 2×10^6 spores/ml.

Virulence tests. Virulence tests were performed on tomato seedlings of the *F. oxysporum* f. sp. *lycopersici* race 1- and 2-resistant tomato variety ‘T15-196’ (genes I and I-2 present). All plants were first grown in a sterilized medium (peat, perlite, and vermiculite, 2:1:1 vol/vol/vol) in plastic pots, until ready for inoculation at the four-true-leaf stage. They were then uprooted, and their roots were gently washed under running tap water. Plantlets were inoculated with the isolates by dipping the roots for 4 to 5 h in spore suspensions (1 to 2×10^6 spores/ml); controls were instituted by dipping the root systems of the plants in distilled water. For each isolate, there were 30 inoculated and 30 control plantlets, each consisting of three replicates of 10 plants per replicate. Subsequently, all plantlets were grown in a hydroponic culture system inside a growth chamber under 60 to 80% relative humidity, 12 h of light (5,000 lux) at 22°C, and 12 h of darkness at 20°C.

Approximately 10 days after inoculation, disease severity was recorded based on a 0 to 4 severity rating scale, with 0 = no disease

symptoms; 1 = light or moderate brown lesions on taproots and lateral roots, without rot; 2 = light or moderate rot of taproot, new secondary roots present, no disease symptoms on the leaves; 3 = light or moderate wilting of leaves, stunting, severe rot on taproot and lateral roots, no new secondary roots, crown rot, and vascular discoloration in the stem; and 4 = stem bases constricted into lines, dead or almost dead plantlets (Hibar et al. 2007; Manzo et al. 2016; Reis and Boiteux 2007; Vakalounakis and Fragkiadakis 1999).

The percentage of disease index (DI) was calculated via the following formula (Sharma et al. 2018):

$$DI = \left[\frac{\sum (\text{scale} \times \text{number of plants in that scale})}{(\text{highest scale} \times \text{total number of assessed plants})} \right] \times 100$$

According to their virulence grade (expressed by the DI), isolates were then classified into four categories: nonvirulence (N), DI = 0 to 10; low virulence (L), DI = 11 to 30; moderate virulence (M), DI = 31 to 60; and high virulence (H), DI = 61 to 100 (Bertoldo et al. 2015).

DNA extraction. For extraction of genomic DNA, the isolates were cultured on PDA at 25°C for 14 days. Genomic DNA was extracted and stored in sterile distilled water at –20°C.

Molecular identification. The isolates were identified according to the method of Hirano and Arie (2006), with four different primer sets, uni, sp13, sp23, and sprl, which amplified DNA bands of 670, 445, 518, and 947 bp, respectively. These primers were able to discriminate all three *F. oxysporum* f. sp. *lycopersici* races as well as *F. oxysporum* f. sp. *radicis-lycopersici*. Details of these primers are given in Table 1. The uni primer set was used to differentiate *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici* from other *formae speciales* of *F. oxysporum*. The sp13 primer set amplified a fragment from *F. oxysporum* f. sp. *lycopersici* races 1 and 3. The sp23 primer set amplified a fragment from *F. oxysporum* f. sp. *lycopersici* races 2 and 3 and differentiated *F. oxysporum* f. sp. *lycopersici* races 2 and 3 from *F. oxysporum* f. sp. *lycopersici* race 1 and *F. oxysporum* f. sp. *radicis-lycopersici*. The sp13 and sp23 primers confirmed the identity of *F. oxysporum* f. sp. *lycopersici* race 3, and the primer sprl was used to detect *F. oxysporum* f. sp. *radicis-lycopersici*. The PCR reaction mixtures were prepared in a total volume of 10 µl containing 10 to 20 ng of DNA template, 1 µl of 10× Taq buffer (20 mM Mg²⁺ plus), 0.25 mM of each forward and reverse primer, 0.2 mM of dNTPs, and 0.05 units of Taq DNA polymerase (TaKaRa, Code No. RR001A). The PCR conditions were as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 60 s, annealing at 62°C for 60 s, and elongation at 72°C for 90 s; and a final elongation at 72°C for 10 min. The amplification products were separated with 1.2% agarose gel electrophoresis in Tris-borate-EDTA buffer at 150 V for 30 min. DL2000 Marker (Takara Biotechnology Co. Ltd., Dalian, China) was used as a molecular weight standard. The DNA bands were stained with DNA_{GREEN} (UV) (Tiandz Gene Technology Co. Ltd., Beijing, China), visualized under ultraviolet light, and photographed with a UVI Gel Documentation System (UVItec Limited Cambridge, UK) (Fig. 1).

Table 1. Primers used in this study and their sequences

Primer name	Primer sequence (5'–3')	Target	Reference
pgx4-F	CTGCCCGCTGGGAACAAGCT	<i>pgx4</i>	
pgx4-R	CTTAACCGTTGAACCTTCTAAC	<i>pgx4</i>	
ITS1	TCCGTAGGTGAACCTGCGG	ITS	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	ITS	White et al. (1990)
uniF	ATCATCTTGTGCCAAGCTTCAG	<i>pgl</i>	Hirano and Arie (2006)
uniR	GTTTGTGATCTTTGAGTTGCCA	<i>pgl</i>	Hirano and Arie (2006)
sprl-F	GATGGTGGAAACGGTATGACC	<i>pgx4</i>	Hirano and Arie (2006)
sprl-R	CCATCACACAAGAACACAGGA	<i>pgx4</i>	Hirano and Arie (2006)
sp13-F	GTCAGTCCATTGGCTCTCTC	<i>pgx4</i>	Hirano and Arie (2006)
sp13-R	TCCTTGACACCATCAGAG	<i>pgx4</i>	Hirano and Arie (2006)
sp23-F	CCTCTGTCTTTGTCTCACGA	<i>pgl</i>	Hirano and Arie (2006)
sp23-R	GCAACAGGTCGTGGGAAAA	<i>pgl</i>	Hirano and Arie (2006)

PCR amplification and sequencing. Molecular analysis was further performed by sequencing the ITS region and the exopolygalacturonase gene (*pgx4*).

The ITS regions of the 91 isolates were amplified with the specific primer pairs ITS1/ITS4 shown in Table 1 (White et al. 1990). The reaction mixtures were prepared as described above. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. The products were again confirmed via electrophoresis on a 1.2% agarose gel.

The *F. oxysporum pgx4* gene was amplified with primers *pgx4-F* and *pgx4-R*. The *pgx4-F/pgx4-R* primer set (Table 1) was designed based on GenBank *Fusarium* spp. sequences (GenBank accession numbers AB256795, AB256796, AB256820, AB256798, AF136444, AB256809, AB256805, and AB256835). The PCR

conditions were as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 60 s, and elongation at 72°C for 90 s; and a final elongation at 72°C for 10 min.

The ITS and *pgx4* PCR products were purified with a TaKaRa MiniBEST DNA Fragment Purification Kit (Takara Biotechnology Co. Ltd., Dalian, China), cloned into the pMD18-T vector (Takara, Dalian, China), and then sequenced (Sangon Biotech Co. Ltd., Shanghai, China). The sequences were edited, vector sequences removed, and assembly was performed in Chromas software (Technelysium Pty Ltd, Tewantin QLD, Australia). Edited sequences were used for subsequent phylogenetic analysis.

Phylogenetic analysis. Similarity searches were performed with the BLASTn program for the ITS sequences of all isolates and *pgx4* sequences of *F. oxysporum* isolates; 15 ITS sequences and 9 *pgx4* sequences downloaded from GenBank were considered as controls (Table 2). Multiple sequences were aligned with the ClustalW program of MEGA 7.0 software (Pennsylvania State University, University Park, PA, USA) (Kumar et al. 2016); phylogenetic trees were constructed via the neighbor-joining (NJ) method with standard bootstrapping (1,000 replicates) in MEGA 7.0 software. The alignment results were rendered with TexShade software (Biology Workbench) (Beitz 2000). Homology comparisons of the *pgx4* sequences were performed with DNAMAN version 9.0 software (Lynnon Corporation, Quebec, Canada), and genetic diversity was analyzed.

Results

Virulence tests. Based on the calculated DI, 11 nonvirulent isolates, 14 low virulent isolates, 11 moderately virulent isolates, and 55 highly virulent isolates were detected (Table 3).

Molecular identification. *Formae speciales* and races of *F. oxysporum* were identified with the primer sets described by Hirano and Arie (2006). Primer set uni amplified a 670- to 672-bp fragment and differentiated *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici* from other *formae speciales* of *F. oxysporum* (Fig. 1A). Primer set sp13 amplified a 445-bp fragment from *F. oxysporum* f. sp. *lycopersici* races 1 and 3 (Fig. 1B) but did not amplify any fragment from race 2 and *F. oxysporum* f. sp. *radicis-lycopersici*. Primer set sp23 amplified a 518-bp fragment from *F. oxysporum* f. sp. *lycopersici* races 2 and 3 (Fig. 1C) but did not amplify any fragment from race 1 and *F. oxysporum* f. sp. *radicis-lycopersici*. The

Fig. 1. PCR products of four specific primer sets used to detect some isolates obtained from wilt and root rot diseases of tomato in China. **A**, Uni (670 bp) for differentiation *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* from other *formae speciales* of *F. oxysporum*; **B**, sp13 (445 bp) for *F. oxysporum* f. sp. *lycopersici* races 1 and 3; **C**, sp23 (518 bp) for *F. oxysporum* f. sp. *lycopersici* races 2 and 3; and **D**, sp1 (947 bp) for *F. oxysporum* f. sp. *radicis-lycopersici*. All PCR products were electrophoresed in a 1.2% agarose gel.

Table 2. Isolates used as controls in this study, their origins, genes, GenBank accession numbers, species, *formae speciales*, and races

Isolate	GenBank accession no.	Origin	Gene	Length (bp)	Species, <i>formae speciales</i> (f. sp.), race
Saitama ly1	AB256795	Japan	<i>pgx4</i>	1,873	f. sp. <i>lycopersici</i> race 1
MAFF103037	AB256816	Japan	<i>pgx4</i>	1,873	f. sp. <i>lycopersici</i> race 1
Saitama ly2	AB256796	Japan	<i>pgx4</i>	1,873	f. sp. <i>lycopersici</i> race 2
MAFF103038	AB256818	Japan	<i>pgx4</i>	1,873	f. sp. <i>lycopersici</i> race 2
FTOfO9701	AB256797	Japan	<i>pgx4</i>	1,873	f. sp. <i>lycopersici</i> race 3
02MT-1	AB256820	Japan	<i>pgx4</i>	1,873	f. sp. <i>lycopersici</i> race 3
Saitama rly	AB256798	Japan	<i>pgx4</i>	1,873	f. sp. <i>radicis-lycopersici</i>
MAFF103045	AB256831	Japan	<i>pgx4</i>	1,873	f. sp. <i>radicis-lycopersici</i>
	AF136444	Spain	<i>pgx4</i>	2,801	f. sp. <i>radicis-lycopersici</i>
G303	MH681160	Poland	ITS	544	<i>Fusarium oxysporum</i>
MF22472	MH911412	India	ITS	545	<i>F. oxysporum</i>
ELRF 7	KX786246	USA	ITS	545	<i>F. oxysporum</i>
KACC 48307	MG356946	South Korea	ITS	544	<i>F. oxysporum</i>
ATCC 52429	DQ452455	South Korea	ITS	544	f. sp. <i>radicis-lycopersici</i>
APBSWTPF101	MG569656	India	ITS	546	<i>Fusarium equiseti</i>
Fg389	MH681145	Netherlands	ITS	545	<i>Fusarium graminearum</i>
FsDAG11	KX583231	Mexico	ITS	571	<i>Fusarium solani</i>
AV1	MH517359	India	ITS	570	<i>F. solani</i>
BLH-W1	MG836251	China	ITS	566	<i>F. solani</i>
F8	HQ380763	China	ITS	558	<i>Fusarium proliferatum</i>
MF11	MH911375	India	ITS	560	<i>F. oxysporum</i>
SA340	KX953613	Mexico	ITS	545	<i>Ascomycota</i> sp.
11	EU594566	China	ITS	558	<i>Plectosphaerella cucumerina</i>
FJTP01	KJ162355	China	ITS	866	<i>Pythium aphanidermatum</i>

specific primer set *sp1* amplified a 947-bp fragment from *F. oxysporum* f. sp. *radicis-lycopersici* (Fig. 1D). Seven isolates were identified as *F. oxysporum* f. sp. *lycopersici* race 1, 2 isolates as *F. oxysporum* f. sp. *lycopersici* race 2, 35 isolates as *F. oxysporum* f. sp. *lycopersici* race 3, and 20 isolates as *F. oxysporum* f. sp. *radicis-lycopersici* (Table 3).

ITS amplification and sequence analysis. The ITS regions of the isolates were amplified with ITS1/ITS4 primers and sequenced; these sequences ranged from 544 to 866 bp in length. Length polymorphisms of the ITS fragments from different species were observed at the sequence level, as follows: *F. oxysporum*, 544, 545, and

Table 3. Identification of isolates collected from diseased tomato plants in China with specific primers according to Hirano and Arie (2006) and virulence grade based on virulence test^a

Geographic origin	Isolate	uni	sp13	sp23	sp1	pgx4	Formae speciales or race ^b	Disease index	Virulence grade
Haining, Zhejiang	HN-R-C-16	–	–	–	–	–		12.3	L
	HN-R-C-1	–	–	–	–	–		8.1	N
	HN-R-C-9	–	–	–	–	–		2.3	N
	HN-R-C-13	–	–	–	–	–		15.7	L
	HN-R-9	–	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	99	H
	HN-W-15	–	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	95.7	H
	HN-R-8	–	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	90.1	H
	HN-W-13	–	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	86.67	H
	HN-R-4	–	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	98.2	H
	HN-W-2	–	–	–	–	–		18	L
	HN-W-6	–	–	–	–	–		21.4	L
	HN-W-1	–	–	–	–	–		24.1	L
	HN-W-12	–	–	–	–	–		8.9	N
	HN-W-11	–	–	–	–	–		5.3	N
	HN-R-3	+	+	+	–	+	Race 3	100	H
	HN-R-1	+	+	+	–	+	Race 3	100	H
	HN-R-2d	+	+	+	–	+	Race 3	100	H
	HN-R-5	+	+	+	–	+	Race 3	100	H
	HN-W-3	+	+	+	–	+	Race 3	100	H
	HN-W-5	+	+	+	–	+	Race 3	100	H
	HN-W-8	+	+	+	–	+	Race 3	100	H
	HN-W-14	+	+	+	–	+	Race 3	100	H
Zibo, Shandong	ZB-38	–	–	–	–	–		3.2	N
	ZB-34	–	–	–	–	–		6.9	N
	ZB-23	–	–	–	–	–		0	N
	ZB-13	–	–	–	–	–		0	N
	ZB-12	–	–	–	–	–		0	N
	ZB-8	–	–	–	–	–		0	N
	ZB-9	–	–	–	–	–		0	N
	ZB-6	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	88.5	H
	ZB-18	+	+	+	–	+	Race 3	100	H
	ZB-36	+	+	+	–	+	Race 3	100	H
	ZB-27	+	+	+	–	+	Race 3	100	H
	ZB-40	+	+	+	–	+	Race 3	100	H
	ZB-4	+	+	+	–	+	Race 3	100	H
	ZB-15	+	+	+	–	+	Race 3	100	H
	ZB-3	+	+	+	–	+	Race 3	97.5	H
	ZB-11	+	+	+	–	+	Race 3	100	H
	ZB-2	+	+	+	–	+	Race 3	100	H
	ZB-1	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	90.2	H
	ZB-5	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	85.3	H
	ZB-32	+	+	+	–	+	Race 3	100	H
Xi'an, Shanxi	XA-10	–	–	–	–	–		35	M
	XA-3	–	–	–	–	–		37.5	M
	XA-11	–	–	–	–	–		35	M
	XA-W-2	+	+	+	–	+	Race 3	100	H
	XA-5	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	97.5	H
	XA-8	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	100	H
	XA-13	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	88.64	H
	XA-1	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	100	H
	XA-4	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	100	H
	XA-6	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	100	H
	XA-7	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	87.5	H
	XA-2	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	81.1	H
	XA-9	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	100	H
	XA-12	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	87.5	H
	XA-W-4	+	+	+	–	+	Race 3	91.7	H

(Continued on next page)

^a –, no DNA bands; +, produce clear DNA bands in agarose gel.

^b Race: *Fusarium oxysporum* f. sp. *lycopersici* race.

558 bp; *Fusarium solani*, 566 and 568 bp; *Fusarium proliferatum*, 558 bp; *Fusarium graminearum*, 545 bp; *Fusarium equiseti*, 546 bp; *Pythium aphanidermatum*, 866 bp; *Ascomycota* sp., 601 bp; and *Plectosphaerella cucumerina*, 554 bp. *F. oxysporum* and *F. solani* revealed within-species polymorphisms. Thus, different species of the genus *Fusarium* were further analyzed. Eight representative sequences were selected to perform the alignment. The alignment for the ITS region comprised 575 bp, of which 479 were conserved, 94 were variable, 78 were parsimony-informative sites, and 15 were parsimony singleton sites. We found that most of the length variations in the ITS sequences (including insertion/deletion differences) were present between the spacers; the 18S, 5.8S, and 28S regions were more highly conserved. However, some variation points between *F. solani* and other *Fusarium* species were identified in the 5.8S region and 28S motif. Three variation sites (203, 228, and 496) were present (Fig. 2). However, the *P. aphanidermatum*, *P. cucumerina*, and *Ascomycota* sp. ITS regions were so highly divergent from the other sequences that a general alignment was not possible.

ITS phylogeny. The BLASTn search for similarities indicated 99 to 100% identity. To reveal phylogenetic relationships, 15 closely related ITS sequences (Table 2) were used to construct a phylogenetic tree based on the search results. The BLASTn analysis identified the species as *F. oxysporum* (70.33%, 64 isolates), *F. solani* (12.09%, 11 isolates), *F. proliferatum* (2.20%, 2 isolates), *F. graminearum* (2.20%, 2 isolates), *F. equiseti* (1.10%, 1 isolate), *P. aphanidermatum* (6.60%, 6 isolates), *Ascomycota* sp. (2.20%, 2 isolates), and *P. cucumerina* (3.30%, 3 isolates) (Fig. 3). According to our data, 87.91% of the isolates corresponded to the genus *Fusarium*. Based on ITS sequences, these isolates were divided into seven (I to VII) major clades (Fig. 4). Clades I, II, III, and IV included a higher proportion of isolates. *F. oxysporum* isolates clustered into two distinct

clades (I and IV), comprising the most virulent isolates of the ‘T15-196’ tomato variety, whereas the nonvirulent isolates were found in clades II, III, V, and VII. Isolates of *F. oxysporum* HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8 were found in clade IV; our phylogenetic analyses of the rDNA-ITS region revealed that these isolates were evolutionarily distant from *F. oxysporum* and more closely related to *F. proliferatum*. Isolates from *F. graminearum* clustered together with *F. equiseti* in clade II. The other species each occupied individual clades.

Pgx4 phylogeny. The PCR products generated with pgx4-F and pgx4-R primers were sequenced, and a phylogenetic analysis of *F. oxysporum* based on the *pgx4* sequences was performed. We found that 64 *F. oxysporum* isolates isolated from roots of tomato plants with wilt and root rot disease symptoms in China and nine closely related sequences retrieved from a BLASTn search grouped into six major clades (A to F) (Fig. 5). The *F. oxysporum* f. sp. *lycopersici* race 3 isolates from China belonged to clade A, together with race 3 from Japan. The *F. oxysporum* f. sp. *lycopersici* races 1 from Japan and China did not cluster together and formed independent clades B and C, respectively. Clade D uniquely consisted of *F. oxysporum* f. sp. *radicis-lycopersici* isolates. Clade E contained a mixture of isolates, including SY-2-1 and SY-2-7 isolates from Hainan province, as well as *F. oxysporum* f. sp. *lycopersici* race 2 from Japan and China. Clade F consisted of HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8 isolates. SY-2-1, SY-2-7, HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8 were unknown *formae speciales* or races of *F. oxysporum*.

Homology analysis. Based on the phylogenetic tree of the *pgx4* sequences of *F. oxysporum* isolates, 10 representative sequences were selected for genetic diversity analysis, and their nucleotide sequences were subjected to homology alignment (Table 4). The

Table 3. (Continued from previous page)

Geographic origin	Isolate	uni	sp13	sp23	spri	pgx4	Formae speciales or race ^b	Disease index	Virulence grade
Sanya, Hainan	SY-2-8	–	–	–	–	+		83.33	H
	SY-2-3	–	–	–	–	+		89.8	H
	SY-2-7	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	68.75	H
	SY-1-7	–	–	–	–	–		46.25	M
	SY-3-1	–	–	–	–	–		40.5	M
	SY-2-15	–	–	–	–	–		37.9	M
	SY-1-3	–	–	–	–	–		33.5	M
	SY-2-1	+	–	–	+	+	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	75.32	H
	SY-2-20	+	+	+	–	+	Race 3	83.5	H
	SY-1-2	+	+	+	–	+	Race 3	75	H
	SY-2-16	+	+	–	–	+	Race 1	25.9	L
	SY-2-17	+	+	–	–	+	Race 1	20.5	L
	SY-2-9	+	+	–	–	+	Race 1	23.8	L
	SY-2-14	+	+	–	–	+	Race 1	14.3	L
Jinhua, Zhejiang	JH-14	–	–	–	–	–		21.2	L
	JH-18	–	–	–	–	–		27.9	L
	JH-16	+	–	+	+	+	Race 2	57.8	M
	JH-19	+	–	+	+	+	Race 2	49.3	M
	JH-17	+	+	–	–	+	Race 1	18.3	L
	JH-20	+	+	–	–	+	Race 1	11.2	L
	JH-15	+	+	–	–	+	Race 1	24.9	L
Shouguang, Shandong	SG-2-1	+	+	+	–	+	Race 3	80.3	H
	SG-2-7	+	+	+	–	+	Race 3	79.6	H
	SG-1-25	+	+	+	–	+	Race 3	58.8	M
	SG-2-15	+	+	+	–	+	Race 3	83.3	H
	SG-3-25	+	+	+	–	+	Race 3	56.3	M
	SG-3-17	+	+	+	–	+	Race 3	80.7	H
	SG-2-21	+	+	+	–	+	Race 3	100	H
Xiaoshan, Zhejiang	XS-22	+	+	+	–	+	Race 3	100	H
	XS-4	+	+	+	–	+	Race 3	100	H
	XS-19	+	+	+	–	+	Race 3	100	H
	XS-1-5	+	+	+	–	+	Race 3	100	H
	XS-1-32	+	+	+	–	+	Race 3	100	H
	XS-1-3	+	+	+	–	+	Race 3	100	H

highest similarity level found was 99.9%; the lowest similarity level, 95.3%, was detected between HN-W-13 and JH-16 sequences. *Fusarium oxysporum* f. sp. *lycopersici* races 1, 2, and 3 and *F. oxysporum* f. sp. *radicis-lycopersici* from Chinese and Japanese isolates were highly homologous, with >98% identity. *F. oxysporum* f. sp. *lycopersici* races 2 and 3 and *F. oxysporum* f. sp. *radicis-lycopersici* from China shared 99.9% similarity with *F. oxysporum* f. sp. *lycopersici* races 2 and 3 and *F. oxysporum* f. sp. *radicis-lycopersici* from Japan. *F. oxysporum* f. sp. *lycopersici* race 1 from China had 99.7% similarity with *F. oxysporum* f. sp. *lycopersici* race 1 from Japan. In addition, HN-W-13 had only 95% similarity with other *F. oxysporum* isolates (Table 4).

Geographic distribution of the isolates. In this study, 91 fungal isolates from diseased tomato plants were collected from 2016 to 2018 at seven geographic regions in China (Xiaoshan, Haining, Jinhua, Sanya, Zibo, Shouguang, and Xi'an) and further identified. More than one strain type was found in diseased plants obtained from five of these locations. Six strain types were detected in Zibo, five in Sanya, four in Haining, and three in Jinhua and Xi'an. Only one strain type was recognized in Xiaoshan; the same occurred in Shouguang. *F. oxysporum* f. sp. *lycopersici* race 1, *F. oxysporum* f. sp. *lycopersici* race 3, *F. oxysporum* f. sp. *radicis-lycopersici*, *F. solani*, and *P. aphanidermatum* were found in diseased plants from at least two different locations (Fig. 6). The distribution of the isolates in different areas was thus not regular, and the regional characteristics were not obvious.

Based on the results of virulence tests, the main pathogens in each of these regions may be described as follows: *F. oxysporum* f. sp. *lycopersici* race 3 in Xiaoshan and Shouguang; *F. oxysporum* f. sp. *lycopersici* race 3 and *F. oxysporum* f. sp. *radicis-lycopersici* in Xi'an and Zibo; *F. oxysporum* f. sp. *lycopersici* race 3 and other unknown *formae speciales* and races of *F. oxysporum* in Haining; *F.*

oxysporum f. sp. *lycopersici* race 2 in Jinhua; and *F. oxysporum* f. sp. *lycopersici* race 3, *F. proliferatum*, and other unknown *formae speciales* and races of *F. oxysporum* in Sanya. *F. oxysporum* f. sp. *lycopersici* race 3 was found to be widespread and highly virulent in the analyzed areas except for Jinhua. *F. oxysporum* f. sp. *lycopersici* race 3 and *F. oxysporum* f. sp. *radicis-lycopersici* were the predominant pathogens associated with tomato wilt and root rot diseases.

Discussion

Previous studies have shown that in addition to *F. oxysporum*, other species can cause wilt and root rot symptoms (Chang et al. 2018; Hyder et al. 2019; Lievens et al. 2004). The results from the present study support these findings. In this study, except for the dominant species of *F. oxysporum* (70.33%), *F. solani*, *F. proliferatum*, *F. graminearum*, *F. equiseti*, *P. aphanidermatum*, *Ascomycota*

Fig. 3. Quantitative distribution of the isolate types detected in diseased tomato plants in China.

Fig. 2. Comparison of ribosomal DNA-internal transcribed spacer sequences between different species of the genus *Fusarium* collected from diseased tomato in China. Dots denote identities; dashes denote deletions. The variation points between *Fusarium solani* and other *Fusarium* species, marked with red boxes and emphasized with black triangles, locate at 5.8S region and 28S motif.

sp., and *P. cucumerina* could induce similar symptoms of wilt and root rot diseases in tomato, but their frequency was lower, and their virulence was weaker than that of *F. oxysporum*.

The physiological races of *F. oxysporum* could be effectively differentiated by sequence analysis of a few genes (Lievens et al. 2009; Mes et al. 1999). The PCR-based technique provides primary advantages for the identification of the *formae speciales* and races of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* (Baysal et al. 2009; Çolak and Biçici 2013; Hirano and Arie 2006). PCR products using the primer sets uni, sp13, sp23, and sprl were generated only from *F. oxysporum* and not from the other species.

The *pgx4* gene is sufficiently representative to be used to compare *F. oxysporum* isolates (Hirano and Arie 2009). A fragment of approximately 1,812 to 1,813 bp was successfully amplified. To infer phylogenetic relationships between *F. oxysporum* isolates, we constructed a phylogenetic tree based on sequences of the *pgx4* gene, which led to the classification of the *F. oxysporum* isolates obtained from symptomatic tomato plants into six clades. The number of *F. oxysporum* members in each clade ranged from 2 to 37. SY-2-1 and SY-2-7 isolates clustered together with *F. oxysporum* f. sp. *lycopersici* race 2 in clade E. HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8 isolates did not cluster together with *F. oxysporum* f. sp. *radicis-lycopersici*, and they formed the independent clade F.

We also observed some discrepancies with regard to the isolates SY-2-1, SY-2-7, HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8, which were determined to be *F. oxysporum* f. sp. *radicis-lycopersici* according to the method of Hirano and Arie (2006), but they were identified only as *F. oxysporum* based on *pgx4* sequences. Homology alignment results showed that HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8 isolates had only 95.6% similarity with *F. oxysporum* f. sp. *radicis-lycopersici* (AB256798) from Japan. The degree of homologous variation among the sequences was large. The isolates HN-W-15, HN-R-9, HN-R-4, HN-W-13, and HN-R-8 were not *F. oxysporum* f. sp. *radicis-lycopersici*. The SY-2-1 and SY-2-7 isolates shared 99.1% similarity with *F. oxysporum* f. sp. *lycopersici* race 2 (AB256796) from Japan, but the sp23 primers did not amplify a fragment from race 2. Primers sp23 and sprl separately amplified 518-bp and 947-bp fragments from isolates JH-16 and JH-19, respectively, which were further identified as *F. oxysporum* f. sp. *lycopersici* race 2 by *pgx4* sequence analysis. Thus, different *formae speciales* and races of *F. oxysporum* were accurately identified by molecular identification carried out according to the method of Hirano and Arie (2006) and the *pgx4* sequence-based phylogenetic tree and homology alignments. In this study, 70.33% of the total isolates corresponded to *F. oxysporum*, which comprised *F. oxysporum* f. sp. *lycopersici* race 3 (38.46%, 35 isolates), *F. oxysporum* f. sp. *radicis-lycopersici* (14.29%, 13 isolates), *F. oxysporum* f. sp. *lycopersici*

Fig. 4. Phylogenetic analysis of ribosomal DNA–internal transcribed spacer sequences of pathogens associated with tomato wilt and root rot in China by the neighbor-joining method.

Fig. 5. Phylogenetic tree based on the *pgx4* sequences of *Fusarium oxysporum* isolates obtained from diseased tomato in China. The tree was constructed by the neighbor-joining method in MEGA version 7.0.

Table 4. Homology analysis of representative sequences of *Fusarium oxysporum* isolates from China and Japan^a

No.	Origin	Isolate	1	2	3	4	5	6	7	8	9	10
1	Japan	Saitama ly1	100									
2	China	SY-2-16	99.7	100								
3	Japan	Saitama ly2	98.7	98.7	100							
4	China	JH-16	98.6	98.6	99.9	100						
5	Japan	FToFO9701	99.7	99.5	98.9	98.8	100					
6	China	HN-R-1	99.7	99.5	98.9	98.8	99.9	100				
7	Japan	Saitama rly	99.4	99.3	98.9	98.8	99.4	99.4	100			
8	China	XA-6	99.3	99.2	98.8	98.7	99.4	99.3	99.9	100		
9	China	SY-2-7	98.9	98.9	99.1	99.0	98.7	98.7	98.8	98.7	100	
10	China	HN-W-13	95.5	95.5	95.4	95.3	95.4	95.4	95.6	95.5	95.5	100

^a Values express percentage of homology.

Fig. 6. Isolate types identified in diseased tomato plants from seven geographic regions. More than one isolate type was found in samples from five locations, and *Fusarium oxysporum* f. sp. *lycopersici* race 3 was identified in diseased plants from all sites other than Jinhua. Several isolate types were found in diseased plants from a single location.

race 1 (7.69%, 7 isolates), *F. oxysporum* f. sp. *lycopersici* race 2 (2.20%, 2 isolates), and other *F. oxysporum* isolates (7.69%) in Figure 3. The other *F. oxysporum* isolates could be additional *formae speciales* or new physiological races, which need to be further explored.

F. oxysporum f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* have been previously reported in China, with *F. oxysporum* f. sp. *lycopersici* race 1 being the predominant race and *F. oxysporum* f. sp. *lycopersici* races 2 and 3 rarely occurring. Race 1 of *F. oxysporum* f. sp. *lycopersici* was originally reported from Beijing and Hubei, China in 1982 (Chen 1982) and has been subsequently observed in many other regions, including Shandong, Sichuan, Heilongjiang, and Shanxi (Li et al. 1991; Wang et al. 1996; Zheng and Wang 2000). *F. oxysporum* f. sp. *radicis-lycopersici* was first reported in Beijing in 2012 (Geng et al. 2012) but has since been reported in Shandong (Cheng et al. 2016; Li et al. 2018) and Hebei (Zhang et al. 2017). Present pathogens have overcome the resistance to *F. oxysporum* f. sp. *lycopersici* race 1 previously demonstrated by tomato varieties in China.

The results of the present study thus demonstrate that race 3 of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* are the major pathogens responsible for wilt and root rot of tomato in different regions of China. The widely used varieties of fresh market tomato appear to have no resistance against *F. oxysporum* f. sp. *lycopersici* race 3 and *F. oxysporum* f. sp. *radicis-lycopersici*, which are highly virulent to tomato plants. Therefore, the development of tomato varieties with resistance against *F. oxysporum* f. sp. *lycopersici* race 3 and *F. oxysporum* f. sp. *radicis-lycopersici* is important as a disease management strategy.

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