Notes

Specific PCR Detection of Four Quarantine *Fusarium* Species in Korea

Sae-Yeon Hong¹, Mi Ran Kang¹, Eun-Ji Cho¹, Hee-Kyoung Kim^{1,2} and Sung-Hwan Yun^{1*}

¹Department of Medical Biotechnology, and ²Research Institute for Basic Sciences, Soonchunhyang University, Asan 336-745, Korea

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Fusarium species, a large group of plant pathogens, potentially pose quarantine concerns worldwide. Here, we focus on the development of a method for detecting four *Fusarium* species in quarantined plants in Korea: *F. solani* f. sp. *cucurbitae*, *F. stilboides*, *F. redolens*, and *F. semitectum* var. *majus*. Species-specific primers were designed from the nucleotide sequences of either the translation elongation factor-1 alpha (*TEF1*) gene or RNA polymerase II subunit (*RPB2*) gene. Two different primer sets derived from *TEF1*, all specific to *F. solani* f. sp. *cucurbitae*, were able to differentiate the two races (1 and 2) of this species. A set of nested primers for each race was designed to confirm the PCR results. Similarly, two primer sets derived from *RPB2* successfully amplified specific fragments from five *F. stilboides* isolates grouped within a single phylogenetic clade. A specific *TEF1* primer set amplified a DNA fragment from only four of the 12 *F. redolens* strains examined, which were grouped within a single phylogenetic clade. All of the *F. semitectum* var. *majus* isolates could be specifically detected with a single *RPB2* primer set. The specificity of the primer sets developed here was confirmed using a total of 130 *Fusarium* isolates.

Keywords : *Fusarium*, PCR detection, quarantine fungi, specific primers

Fusarium is a large genus of filamentous fungi that includes a broad range of plant pathogens causing wilt and crown and root rot in a variety of hosts. Some members of this genus also produce various toxic secondary metabolites such as mycotoxins, which are harmful to both human and plant health. Due to the diversity of plant diseases caused by *Fusarium* species, these species potentially pose quarantine concerns worldwide. In Korea, eleven pathogenic *Fusarium* species are listed as quarantine pests (*F. solani* f. sp. *cucurbitae*, *F. stilboides*, *F. semitectum* var. *majus*, *F. oxysporum* var. *redolens*, *F. oxysporum* f. sp. *cattleyae*, *F. oxysporum* f. sp. *citri*, *F. oxysporum* f. sp. *cubense*, *F.*

oxysporum f. sp. *radicis-lycopersici*, *F. culmorum*, *F. poae*, and *F. sulphureum*). Here, we report a PCR detection method developed specifically for the first four *Fusarium* species.

Fusarium solani f. sp. *cucurbitae* causes Fusarium crown, fruit, and root rot in cucurbits. This species can be divided into two different races (1 and 2): race 1 (telomorph: *Nectria haematococca* mating population I) causes crown, fruit, and root rot, whereas race 2 (telomorph: *N. haematococca* mating population V) affects only the fruit. Currently, the races are thought to be distinct species, as they are clearly different in terms of sexual mating behavior, other taxonomic characters, and DNA sequence homology (Mehl and Epstein, 2007). *F. stilboides* causes bark and fruit rot of citrus and coffee (Gerlach and Nirenberg, 1982), and is a member of the *Lateritium* clade of *Fusarium*; it is still difficult to distinguish it from some isolates of *F. lateritium*, which also belongs to the *Lateritium* clade (Geiser et al., 2005). *F. semitectum* var. *majus*, a cosmopolitan species, is one of the two varieties of *F. semitectum* (the other is var. *semitectum*) and is regularly associated with a complex of plant diseases (Hawa et al., 2010). *F. redolens* causes wilt, seedling damping-off, and cortical rot and used to be recognized at either the variety or *forma specialis* level of *F. oxysporum* (*F. oxysporum* var. *redolens* or *F. oxysporum* f. sp. *redolens*), but is now known to be distinct from *F. oxysporum* (Bogale et al., 2007). In Korea, the detection of these pathogenic *Fusarium* species in quarantine services relies mostly on the morphology of conidia produced on plant samples, which not only delays the quarantine procedures, but also lead to misidentification of the fungal species. Therefore, it is necessary to develop a fast, reliable method that specifically detects these quarantine species.

The objectives of this study were to 1) confirm the species identification of the *Fusarium* isolates collected for this study using a phylogenetic analysis, 2) design species-specific PCR primer sets for the four quarantine *Fusarium* species, and 3) determine their specificity, sensitivity, and reliability.

Collection of *Fusarium* isolates. For this study, 130 *Fusarium* isolates were collected from several culture col-

*Corresponding author.
Phone) +82-41-530-1288, FAX) +82-41-530-3085
E-mail) sy14@sch.ac.kr

Table 1. *Fusarium* species used in PCR analysis

Isolate	Species	Source
K40031	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i> race 3	KACC
K40032	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race 3	
K40037	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race 2	
K40038	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race 2	
K40046	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race 1	
K40047	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race 1	
K40052	<i>F. oxysporum</i>	
K40053	<i>F. oxysporum</i>	
K41081	<i>F. oxysporum</i>	
K41083	<i>F. oxysporum</i>	
K41087	<i>F. oxysporum</i>	
K41088	<i>F. oxysporum</i>	
K41090	<i>F. oxysporum</i>	
K40236	<i>F. oxysporum</i>	
K44305	<i>F. oxysporum</i>	
K41036	<i>F. semitectum</i>	
K40384	<i>F. solani</i>	
K41092	<i>F. solani</i>	
K41093	<i>F. solani</i>	
K44803	<i>F. acuminatum</i>	
K44819	<i>F. acutatum</i>	
K44820	<i>F. avenaceum</i>	
K42160	<i>F. cerealis</i>	
K42159	<i>F. cerealis</i>	
K44946	<i>F. circinatum</i>	
K44570	<i>F. circinatum</i>	
K44569	<i>F. circinatum</i>	
K42162	<i>F. culmorum</i>	
K42161	<i>F. culmorum</i>	
K41312	<i>F. culmorum</i>	
K44021	<i>F. fujikuroi</i>	
K44019	<i>F. fujikuroi</i>	
K44017	<i>F. fujikuroi</i>	
K44015	<i>F. fujikuroi</i>	
K44010	<i>F. fujikuroi</i>	
K44009	<i>F. fujikuroi</i>	
K44004	<i>F. fujikuroi</i>	
K42687	<i>F. fujikuroi</i>	
K42686	<i>F. fujikuroi</i>	
K42702	<i>F. globosum</i>	
K42701	<i>F. globosum</i>	
K41034	<i>F. lateritium</i>	
K42689	<i>F. nygamai</i>	
K42688	<i>F. nygamai</i>	
K42271	<i>F. oxysporum</i> f. sp. <i>asparagi</i>	
K42273	<i>F. oxysporum</i> f. sp. <i>chrysanthemi</i>	
K42274	<i>F. oxysporum</i> f. sp. <i>ciceris</i>	
K42276	<i>F. oxysporum</i> f. sp. <i>conglutinans</i>	
K42275	<i>F. oxysporum</i> f. sp. <i>glycines</i>	

Table 1. Continued

Isolate	Species	Source
K42795	<i>F. oxysporum</i> f. sp. <i>lactucae</i>	KACC
K42272	<i>F. oxysporum</i> f. sp. <i>cassiae</i>	
K42169	<i>F. oxysporum</i> f. sp. <i>fragariae</i>	
K42172	<i>F. oxysporum</i> f. sp. <i>pisi</i>	
K42173	<i>F. oxysporum</i> f. sp. <i>sesami</i>	
K44025	<i>F. proliferatum</i>	
K44024	<i>F. proliferatum</i>	
K42694	<i>F. proliferatum</i>	
K42693	<i>F. proliferatum</i>	
K42692	<i>F. proliferatum</i>	
K44846	<i>F. pseudograminearum</i>	
K42695	<i>F. sacchari</i>	
K41317	<i>F. sambucinum</i> var. <i>sambucinum</i>	
K43387	<i>F. solani</i>	
K42177	<i>F. solani</i> f. sp. <i>batatas</i>	
K42178	<i>F. solani</i> f. sp. <i>mori</i>	
K42181	<i>F. solani</i> f. sp. <i>pisi</i>	
K42182	<i>F. solani</i> f. sp. <i>robiniae</i>	
K42696	<i>F. subglutinans</i>	
K42852	<i>F. verticillioides</i>	
K42699	<i>F. verticillioides</i>	
K42698	<i>F. verticillioides</i>	
K41321	<i>F. verticillioides</i>	
Z03639	<i>F. graminearum</i>	Dr. J. Leslie, Kansas State Univ., USA
Z03643	<i>F. graminearum</i>	
H48661	<i>F. pseudograminearum</i>	
H11553	<i>F. sporotrichioides</i>	
H11568	<i>F. chlamydosporum</i>	
SNU-lyco	<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	
GS2-2	<i>F. semitectum</i>	
GS2-18	<i>F. semitectum</i>	
F16	<i>F. asiaticum</i> (13818)	
F20	<i>F. boothii</i> (26916)	
F24	<i>F. mesoamericanum</i> (25797)	
F30	<i>F. brasiliense</i> (31281)	
F34	<i>F. meridionale</i> (28723)	CBS
F37	<i>F. lunulosporum</i> (13393)	
F39	<i>F. culmorum</i> (3288)	
C145.44	<i>F. semitectum</i> var. <i>majus</i>	
C161.25	<i>F. semitectum</i> var. <i>majus</i>	
C163.57	<i>F. semitectum</i> var. <i>majus</i>	
C319.73	<i>F. stilboides</i>	FRC
C746.79	<i>F. stilboides</i>	
C101890	<i>F. stilboides</i>	
C101891	<i>F. stilboides</i>	
C101892	<i>F. stilboides</i>	
C115624	<i>F. stilboides</i>	
O0225	<i>F. oxysporum</i> f. sp. <i>cubense</i>	FRC
O1223	<i>F. oxysporum</i> f. sp. <i>cubense</i> race 1	

Table 1. Continued

Isolate	Species	Source
O1564	<i>F. oxysporum</i> f. sp. <i>cubense</i> race 1	FRC
O1565	<i>F. oxysporum</i> f. sp. <i>cubense</i> race 1	
O1566	<i>F. oxysporum</i> f. sp. <i>cubense</i> race 1	
O1567	<i>F. oxysporum</i> f. sp. <i>cubense</i> race 2	
O1568	<i>F. oxysporum</i> f. sp. <i>cubense</i> race 4	
O1955	<i>F. oxysporum</i> f. sp. <i>cubense</i>	
O1968	<i>F. oxysporum</i> f. sp. <i>cubense</i>	
O1090	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O1092	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O1097	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O1101	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O1102	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O1973	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O2022	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O2023	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	
O1140	<i>F. oxysporum</i> var. <i>redolens</i> (<i>F. redolens</i>)	
O1265	<i>F. oxysporum</i> var. <i>redolens</i>	
O1266	<i>F. oxysporum</i> var. <i>redolens</i>	
O1320	<i>F. oxysporum</i> var. <i>redolens</i>	
O1380	<i>F. oxysporum</i> var. <i>redolens</i>	
O1523	<i>F. oxysporum</i> var. <i>redolens</i>	
O1792	<i>F. oxysporum</i> var. <i>redolens</i>	
O1793	<i>F. oxysporum</i> var. <i>redolens</i>	
O1891	<i>F. oxysporum</i> var. <i>redolens</i>	
O1893	<i>F. oxysporum</i> var. <i>redolens</i>	
O1926	<i>F. oxysporum</i> var. <i>redolens</i>	
S0201	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	
S0202	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	
S0203	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	
S0687	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	
S0688	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	
S0696	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	

KACC: Korean Agricultural Culture Collection, Suwon, Korea.
 CBS: the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.
 FRC: *Fusarium* Research Center, University Park, PA, USA.

lections (Table 1). Of these, 44 *Fusarium* isolates of six species [*F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *radicis-lycopersici*, *F. redolens* (*F. oxysporum* var. *redolens*), *F. solani* f. sp. *cucurbitae*, *F. semitectum* var. *majus*, and *F. stilboides*] are listed as quarantine fungi in Korea. The remaining isolates were closely or distantly related species within the genus *Fusarium*. All isolates were stored in 25% glycerol at -80°C and maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). For genomic DNA extraction, they were grown in 50 ml of PD broth at 25°C for 3 days with shaking (150 rpm). Fungal genomic DNA was extracted as described previously (Chi et al., 2009).

Table 2. The nucleotide sequences of *TEF1* from the reference *Fusarium* strains used in the phylogenetic analysis

Strain	Species	Accession # ^a
NRRL25600	<i>F. redolens</i>	AF324294
NRRL26762	<i>F. redolens</i>	AF324313
NRRL28425	<i>F. redolens</i>	AF324308
NRRL28909	<i>F. redolens</i>	AF324318
NRRL 52645	<i>F. redolens</i>	GU250583
40/2.5	<i>F. redolens</i>	DQ854918
60/2.1.1	<i>F. redolens</i>	DQ854922
43412	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986186
43413	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986187
43417	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986191
43419	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986193
43423	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986196
43424	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986197
43425	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	DQ986198
HLM133	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	DQ913751
HLM203	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	DQ913755
HLM258	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	DQ913761
HLM217	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	DQ913756
HLM255	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	DQ913758
HLM256	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	DQ913759
NRRL22142	<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	AF178347
IBT24	<i>F. stilboides</i>	EF526102
IBT7	<i>F. stilboides</i>	EF526101
ISPaVe 1946	<i>F. incarnatum</i> (= <i>F. semitectum</i>)	FN430737
NRRL 31160	<i>F. incarnatum</i> (= <i>F. semitectum</i>)	GQ915510
1559A	<i>F. incarnatum</i> (= <i>F. semitectum</i>)	FJ895279

^a GenBank accession numbers

Phylogenetic analysis. To confirm both the species identification of the collected *Fusarium* isolates and their phylogenetic relationships, partial nucleotide sequences of genes frequently used as DNA markers [those for translation elongation factor 1- α (*TEF1*), RNA polymerase second largest subunit (*RPB2*), beta-tubulin (*TUB1*), and calmodulin M (*calM*)] were amplified from 77 of the collected 130 isolates. In addition, we attempted to amplify a partial fragment of pheromone precursor gene (*PPG1*) from these isolates using a *PPG1*-specific primer set (Kim et al., 2008). The *TEF1* sequences of several *Fusarium* species deposited in the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) (Table 2) were used as reference sequences for a phylogenetic analysis. All primers (Table 3) used for PCR amplification were synthesized by Bioneer Corporation (Daejeon, Korea). Each reaction tube contained 50 ng of template DNA, 1 $\times$ PCR buffer, dNTPs at 0.2 mM each, primers at 10 μM , and 1.25 U ExTaq polymerase (Takara Biomedicals, Shiga, Japan) in 50- μl reaction volumes. The PCR reactions

Table 3. Primers used to amplify DNA markers

Primer	Sequence (5' → 3')	Amplified genes
EF-1	ATGGGTAAGGA(A/G)GACAAGAC	<i>TEF1</i> (O'Donnell et al., 1998)
EF-2	GGA(G/A)GTACCAGT(G/C)ATCATGTT	
tubT1	AACATGCGTGAGATTGTAAGT	<i>TUB1</i> (O'Donnell & Cigelnik, 1997)
tubT22	TCTGGATGTTGTTGGGAATCC	
fRPB2-cF	ATGGG(C/T)AA(A/G)CAAGC(C/T)ATGGG	<i>RPB2</i> (Liu et al., 1999)
fRPB2-11aR	GC(A/G)TGGATCTT(A/G)TC(A/G)TC(C/G)ACC	
CLOX1	CAGCAAAGCATCAGACCACTATAACTC	<i>calM</i> (Mulè et al., 2004)
CLOX2	CTTGTCAGTAACTGGACGTTGGTACT	

were performed with an initial denaturation at 94°C for 2 min, 30 cycles of 94°C (1 min)/50°C or 55°C (1 min)/72°C (2 min), and a final extension at 72°C for 10 min. A 710-bp *TEF1* and ~970-bp *RPB2* fragment were successfully amplified from all 77 isolates examined, whereas the ~530-bp *calM* product was not isolated from six of the 77 isolates. However, a ~710-bp *PPG1* fragment was amplified from only a few isolates, so the attempt to use *PPG1* as a new DNA marker was not successful in this study. All of the amplified products were cloned into pGEMT vector (Promega, Madison, WI, USA), and their nucleotide sequences were determined using primers T7 and SP6 then extended, when needed, using a primer corresponding to the previously determined regions (for *TUB1*). All the nucleotide sequences for each isolate were deposited in the Plant Pathogen DNA Bank (PPDA; <http://ppda.riceblast.snu.ac.kr>) and are available on request. Sequences were edited with Lasergene v6.0 (DNASTAR, Madison, WI, USA) and aligned using ClustalW (Thompson et al., 1994). Maximum parsimony (MP) analyses were performed using MEGA ver. 4.02 (Tamura et al., 2007). The robustness of the MP trees (MPTs) was determined using the full heuristic search option for 1,000 bootstrap replications. The *TEF1* dataset comprised 99 taxa, including 26 from sequence databases with 786 characters, of which 384 were constant, and 317 were parsimony-informative and 402 parsimony-uninformative, generating 481 MPTs. The *RPB2* analysis yielded 267 MPTs from a dataset comprising 986 characters, including 684 uninformative positions and 302 parsimony-informative positions. The *TEF1* and *RPB2* trees had similar topographies that revealed seven strongly supported clades [more than 90% bootstrap support (BS)], each corresponding to the taxonomic positions of most of the collected *Fusarium* isolates (Fig. 1); the topology of the *calM* tree was different from those of the other genes (data not shown). Five of the six *F. stilboides* isolates examined were grouped within a single clade (99% BS), whereas the remaining one (C115624) belonged to the *F. oxysporum* complex clade, suggesting that the species identification of C115624 is incorrect. The three *F. semitectum* var. *majus* isolates ex-

amined were placed in a single clade (99% BS) along with the isolates of the closely related species *F. semitectum*; it is still unclear whether the two *F. semitectum* isolates (GS2-2 and GS2-18) examined and other *F. semitectum* reference strains available in NCBI are distinct from *F. semitectum* var. *majus*. The *F. solani* f. sp. *cucurbitae* race 1 (S0687, S0688, and S0696) and race 2 (S0201, S0202, and S0203) isolates examined here formed two separate clades (99% BS), each along with reference strains, implying that the two races of *F. solani* f. sp. *cucurbitae* are phylogenetically distinct species (Mehl and Epstein, 2007). In addition, the other *F. solani* isolates (K40384, K41092, and K41093), whose *forma speciales* levels have not been identified, grouped within a different subclade, indicating that they are phylogenetically distinct from the *F. solani* f. sp. *cucurbitae* isolates. In contrast to the *Fusarium* species described above, most isolates of the *F. oxysporum* complex resided in a single clade (97% BS), but their *forma speciales* (e.g., *cubense*, *lycopersici*, *radicis-lycopersici*, and *lycocersici*) were not resolved further into strongly supported subclades. This supports the hypothesis that *F. oxysporum* is a complex of morphologically similar fungi, but its *forma speciales* are not separated as reliable taxonomic categories (Baayen et al., 2000; O'Donnell et al., 1998). However, four (O1265, O1266, O1891, and O1893) of the eleven *F. redolens* (synonym: *F. oxysporum* var. *redolens*) examined here resided within a distinct clade, implying that these two taxa are different species (Bogale et al., 2007), although morphology-based diagnoses of *F. redolens* and *F. oxysporum* are still challenging. In this respect, the *F. redolens*-specific clade suggests that the other seven *F. redolens* isolates await further confirmation for species identification.

PCR amplification using species-specific primer sets.

We attempted to design specific primer sets for fungal isolates belonging to four quarantine species (*F. solani* f. sp. *cucurbitae*, *F. stilboides*, *F. semitectum* var. *majus*, and *F. redolens*), each of which resided within a strongly supported clade in the phylogenetic tree (Fig. 1). Based on the

Fig. 1. A phylogenetic tree constructed from the *TEF1* genes of the collected *Fusarium* species using the maximum parsimony (MP) method. The numbers at the nodes indicate bootstrap values estimated from 1,000 replications of the dataset.

Table 4. Specific primers designed in this study

Primer	Sequence (5' → 3')	primer specificity
Fsc1	CGTGATTGGGACGGATGAGAGA	forward primer for <i>F. solani</i> f. sp. <i>cucurbitae</i> (<i>Fsc</i>) race 1
Fsc2	ACGTGAGTGAGAGACATGACGG	forward primer for <i>Fsc</i> race 2
Tsolre1FM	ACATACCAATGACGGTGACATAGTA	forward primer for <i>Fsc</i> races 1&2, and <i>F. redolens</i>
Fsolc1R1	TGGCATCTTGGGCGGGGGGT	reverse primer for <i>Fsc</i> race 1
Fsolc2R1	TTCACAACAGACACTGACTCG	reverse primer for <i>Fsc</i> race 2
Tsol1R	CTGCTTATCTTGGGTCGTGG	reverse primer for <i>Fsc</i> races 1&2, and other <i>F. solani</i> isolates
Tredo1R	TTCGACTCGCCGCTCCCA	reverse primer for <i>F. redolens</i>
SmibolFM	GCAAAAGCCTCTCGCCAC	forward primer for both <i>F. stilboides</i> and <i>F. semitectum</i>
FstR1	GACACCAATAGTACCCTTTTGTC	reverse primer for <i>F. stilboides</i>
Stibo1RM	GTGCTTCATTTCGAGAGTTTG	reverse primer for <i>F. stilboides</i>
Semi1RM	AGGTGTAGAGATATCGCGG	reverse primer for <i>F. semitectum</i>

aligned *TEF1* or *RPB2* nucleotide sequences, we searched variable regions for each species in generating the primer sets (Table 4). Using the *TEF1* sequences, two different primer sets were designed for the PCR detection of *F. solani* f. sp. *cucurbitae* race 1. The primer pairs Fsc1-Tsol1R and Tsolre1FM-Fsolc1R1 (Table 4) amplified 580- and 526-bp fragments, respectively, from genomic DNA of the three race 1 isolates of *F. solani* f. sp. *cucurbitae*; none of the race 2 isolates or the other *Fusarium* isolates including *F. solani* generated the specific amplified fragments (Fig. 2A). In addition, the second primer pair (Tsolre1FM and Fsolc1R1) can be used as a nested primer set using a 100-fold diluted PCR product amplified by the first primer pair as template DNA; no specific amplification was observed from the first PCR products of the isolates of race 2 and other *Fusarium* spp. (Fig. 2A). Similarly, two primer pairs (Fsc2-Tsol1R and Tsolre1FM-Fsolc2R1) were designed for the specific amplification of *F. solani* f. sp. *cucurbitae* race 2. These primer sets successfully amplified 580-

and 174-bp fragments, respectively, from genomic DNA of the race 2 isolates, but not from other fungal isolates, including race 1 and *F. solani*; as in the case of the race 1 isolates, the second primer set can be used as a nested primer set (Fig. 2B). These results support the classification of the two races of *F. solani* f. sp. *cucurbitae* as distinct species (Mehl and Epstein, 2007). PCR with the primer pair Tsolre1FM and Tredo1R amplified a 343-bp fragment from only the four *F. redolens* isolates that reside within a single phylogenetic clade (Fig. 3A). Because the nucleotide sequence of the forward primer (Tsolre1FM) used in this PCR was completely conserved in all of the isolates of *F. solani* f. sp. *cucurbitae* and *F. solani* examined, a PCR fragment specific to each of these isolates could be amplified when using primer Tsol1R, which is specific to both *F. solani* f. sp. *cucurbitae* and *F. solani*, along with this primer. Therefore, multiplex PCR using the three primers (Tsolre1FM, Tsol1R, and Tredo1R) was attempted, and it amplified a specific fragment from either of these two

Fig. 2. Specific amplification of the *TEF1* region from the isolates of *F. solani* f. sp. *cucurbitae* (*Fsc*) race 1 (A) and race 2 (B). (A) Left panel: PCR amplification of a 580-bp fragment from genomic DNA of the *Fsc* race 1 isolates with the primers Fsc1 and Tsol1R. Lanes 1-3: the *Fsc* race 2 isolates (S201, S202, and S203, respectively); 4-6: the *Fsc* race 1 isolates (S687, S688, and S696, respectively); 7-9: the *F. solani* isolates (K40384, K41092, and K41093, respectively); 10-16: other *Fusarium* spp. Middle panel: PCR amplification of a 526-bp fragment from genomic DNA of the *Fsc* race 1 isolates with the primers Tsolre1FM and Fsolc1R1. The lane descriptions are the same as those for the left panel. Right panel: PCR amplification of the 526-bp fragment from the 100-fold-diluted PCR products in the left panel as template DNA with Tsolre1FM and Fsolc1R1. The lane descriptions are the same as those for the left panel. (B) Left: PCR amplification of a 580-bp fragment from genomic DNA of the *Fsc* race 2 isolates with the primers Fsc2 and Tsol1R. Middle: PCR amplification of a 174-bp fragment from genomic DNA of the *Fsc* race 2 isolates with the primers Tsolre1FM and Fsolc2R1. Right: PCR amplification of the 174-bp fragment from the 100-fold diluted PCR products in the left panel as template DNA with Tsolre1FM and Fsolc2R1. The lane descriptions for the three panels are the same as those in (A).

Fig. 3. Specific amplification of the *TEF1* regions from genomic DNA of the *F. redolens* isolates using the primers Tsolre1FM and Tredo1R (A) and from those of the *F. redolens* and *Fsc* isolates using the three primers Tsolre1FM, Tredo1R, and Tsol1R (B). (A) Lanes 1-4: the *F. redolens* isolates (O1265, O1266, O1891, and O1893, respectively); 5-9: other *F. redolens* isolates (O1140, O1265, O1320, O1523, and O1793, respectively); 10-16: other *Fusarium* spp. (B) Lanes 1-2: the *F. solani* isolates (K40384, and K41092, respectively); 3-5: *Fsc* race 2 isolates (S201, S202, and S203, respectively); 6-8: the *Fsc* race 1 isolates (S687, S688, and S696, respectively); 10, 11, 17, 18: the *F. redolens* isolates (O1265, O1266, O1891, and O1893, respectively); 12-16: other *F. redolens* isolates (O1140, O1265, O1320, O1523, and O1793, respectively); 19-22: other *Fusarium* spp.

Fusarium species (*F. redolens* and at least some of the *F. solani* complex) (Fig. 3B). This confirms that these three primers can be used not only to detect both quarantine *Fusarium* species simultaneously, but also to differentiate the two species based on size differences of the PCR products, which is more reliable than PCR detection based on the presence/absence of a specific PCR product. If the multiplex PCR shows positive results, *F. solani* f. sp. *cucurbitae* can be identified further at the race level with the specific primer sets described above (Tsolre1FM-Fsolc1R1 for race 1 and Tsolre1FM-Fsolc2R1 for race 2); no further PCR is necessary for *F. redolens*.

Using the *RPB2* sequences, three primers were designed for the simultaneous PCR detection of both *F. stilboides* and *F. semitectum* var. *majus* (Table 4). One primer (Smibo1FM) was a common forward primer for both species, whereas two reverser primers (Fst1 and Semi1RM) were specific for *F. stilboides* and *F. semitectum* var. *majus*, respectively. These primer sets successfully amplified 578- and 424-bp fragments from the genomic DNA of the isolates of *F. stilboides* and all of the *F. semitectum* var. *majus*, respectively (Fig. 4). The absence of the specific fragment found in *F. stilboides* C115624, which was identified as a member of the *F. oxysporum* complex clade instead of the *F. stilboides* clade in this study (Fig. 1), supports the species confirmation for five of the six *F. stilboides* isolates in the phylogenetic analysis. The only exception was that a DNA fragment was amplified from

Fig. 4. Specific amplification of *TEF1* regions from the isolates of *F. stilboides* and *F. semitectum*. (A) Upper panel: PCR amplification of a 578-bp fragment from genomic DNA of the *F. stilboides* isolates with the primers Smibo1FM and FstR1. Lanes 1-6: the *F. stilboides* isolates (C319.73, C746.79, C101890, C101891, C101892, and C115624, respectively); 7-16: other *Fusarium* spp. Middle panel: PCR amplification of a 263-bp fragment from genomic DNA of the *F. stilboides* isolates with the primers Smibo1FM and Stibo1RM. Bottom panel: PCR amplification of the 263-bp fragment from the 100-fold-diluted PCR products in the upper panel as template DNA with Smibo1FM and Stibo1RM. The lane descriptions for the middle and bottom panels are the same as those for the upper panel. (B) PCR amplification of the 263-bp fragment and a 424-bp fragment from *F. stilboides* and *F. semitectum* var. *majus*, respectively with the three primers Smibo1FM, Semi1RM, and Stibo1RM. Lane 1: *F. semitectum* K41036; 2-4: the *F. semitectum* var. *majus* isolates (C145.44, C161.25, and C163.57, respectively); 5-10: the *F. stilboides* isolates (C319.73, C746.79, C101890, C101891, C101892, and C115624, respectively); 11-16: other *Fusarium* spp.

one *F. lateritium* isolate (data not shown), implying that this isolate is a member of the *F. lateritium* clade, some of which had previously been identified as *F. stilboides* (Geiser et al., 2005). To increase the reliability of the specific PCR detection developed for *F. stilboides*, a nested primer (Stibo1RM) was used along with Smibo1FM to amplify a 263-bp fragment using the 100-fold-diluted PCR product (578 bp) amplified by the primer pair Smibo1FM and as a template (Fig. 4A). Multiplex PCR using the three primers (Smibo1FM, Semi1RM and Stibo1RM) in a single tube also amplified the expected PCR fragments from each species (Fig. 4B). By contrast, the specific PCR primers for *F. semitectum* var. *majus* also amplified the same-size

fragment from some isolates of *F. semitectum*, indicating either that the primer specificity is not sufficient for *F. semitectum* var. *majus*, or that the *F. semitectum* isolates used here need further species confirmation at the intra-specific level. The detection limit for these assays was between 1 and 10 pg fungal genomic DNA per PCR reaction. The specificity of the primer sets developed here was confirmed using a total of 130 *Fusarium* isolates. In addition, the specific primers amplified no fragments from several fungal isolates other than the genus *Fusarium*, which were frequently contaminated in plant samples (data not shown).

To date, primer sets have been designed for the PCR detection of *F. redolens* and each of the *F. solani* f. sp. *cucurbitae* races (Bogale et al., 2007; Mehl and Epstein, 2007), whereas no PCR primers have been developed for *F. stilboides* or *F. semitectum* var. *majus*. All of these previously developed primer sets also specifically amplified isolates of the corresponding *Fusarium* species. Based on the specificity and reliability of the primer sets developed here, they could be used in the PCR detection of the four different quarantine *Fusarium* species in imported plant samples. However, further analyses to increase the specificity and reliability of the PCR primer sets should be done in the near future. Especially, more field isolates of these quarantine *Fusarium* species (e.g., other *forma speciales* of *F. solani* and *F. semitectum* varieties) and closely related isolates must be tested. In addition, it is necessary to determine if the PCR reactions with the genomic DNA of various plant samples are able to amplify the specific PCR products efficiently when they are contaminated with the quarantine *Fusarium* isolates. In this respect, efficient genomic DNA extraction methods from various plant matrixes should be screened. The PCR detection limit of fungal-specific DNA fragments from plant genomic DNA will be also determined.

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References

- Baayen, R. P., O'Donnell, K., Bonants, P. J. M., Cigelnik, E., Kroon, L. P. N. M., Roebroek, E. J. A. and Waalwijk, C. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and non-monophyletic formae speciales causing wilt and rot diseases. *Phytopathology* 90:891-900.
- Bogale, M., Wingfield, B. D., Wingfield, M. J. and Steenkamp, E. T. 2007. Species-specific primers for *Fusarium redolens* and a PCR-RFLP technique to distinguish among three clades of *Fusarium oxysporum*. *FEMS Microbiol. Lett.* 271:27-32.
- Chi, M. H., Park, S. Y. and Lee, Y. H. 2009. A quick and safe method for fungal DNA extraction. *Plant Pathol. J.* 25:108-111.
- Gerlach, W. and Nirenberg, H. I. 1982. The genus *Fusarium*-a pictorial atlas. Mitt Biol Bundesanst Land- Forstwirtschaft, Berlin, Germany.
- Geiser, D. M., Ivey, M. L. L., Hakiza, G., Juba, J. H. and Miller, S. A. 2005. *Gibberella xylarioides* (anamorph: *F. xylarioides*), a causative agent of coffee wilt disease in Africa, is a previously unrecognized member of the *G. fujikuroi* species complex. *Mycologia* 97:191-201.
- Hawa, M. M., Salleh, B. and Latiffah, Z. 2010. Characterization and intraspecific variation of *Fusarium semitectum* (Berkeley and Ravenel) associated with red-fleshed dragon fruit (*Hylocereus polyrhizus* [Weber] Britton and Rose) in Malaysia. *African J. Biotechnol.* 9:273-284.
- Kim, H. K., Lee, T. and Yun, S. H. 2008. A putative pheromone signaling pathway is dispensable for self-fertility in the homothallic ascomycete *Gibberella zeae*. *Fungal Genet. Biol.* 45: 1188-1196.
- Liu, Y. L., Whelen, S. and Hall, B. D. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Mol. Biol. Evol.* 16:1799-1808.
- Mehl, H. L. and Epstein, L. 2007. Identification of *Fusarium solani* f. sp. *cucurbitae* race 1 and race 2 with PCR and production of disease-free pumpkin seeds. *Plant Dis.* 91:1288-1292.
- Mulè, G., Susca, A., Stea, G. and Moretti, A. 2004. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans*. *Eur. J. Plant Pathol.* 110:495-502.
- O'Donnell, K. and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7:103-116.
- O'Donnell, K., Kistler, H. C., Cigelnik, E. and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 95:2044-2049.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.