Specific PCR Detection of Four Quarantine Fusarium Species in 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. stilboideae, 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. stilboideae 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. stilboideae, 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. stilboideae 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-

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**Table 1.** *Fusarium* species used in PCR analysis

| Isolate | Species | Source |
|---------|---------|--------|
| K40031 | *F. oxysporum* f. sp. radicis-lycopersici race 3 | Dr. J. Leslie, Kansas State Univ., USA |
| K40032 | *F. oxysporum* f. sp. lycopersici race 3 | Dr. J. Leslie, Kansas State Univ., USA |
| K40037 | *F. oxysporum* f. sp. lycopersici race 2 | Dr. J. Leslie, Kansas State Univ., USA |
| K40038 | *F. oxysporum* f. sp. lycopersici race 2 | Dr. J. Leslie, Kansas State Univ., USA |
| K40046 | *F. oxysporum* f. sp. lycopersici race 1 | Dr. J. Leslie, Kansas State Univ., USA |
| K40047 | *F. oxysporum* f. sp. lycopersici race 1 | Dr. J. Leslie, Kansas State Univ., USA |
| K40052 | *F. oxysporum* | KACC |
| K40057 | *F. oxysporum* | KACC |
| K40058 | *F. oxysporum* | KACC |
| K41081 | *F. oxysporum* | KACC |
| K41082 | *F. oxysporum* | KACC |
| K41083 | *F. oxysporum* | KACC |
| K41087 | *F. oxysporum* | KACC |
| K41088 | *F. oxysporum* | KACC |
| K41090 | *F. oxysporum* | KACC |
| K40236 | *F. oxysporum* | KACC |
| K44305 | *F. oxysporum* | KACC |
| K44803 | *F. acuminatum* | KACC |
| K44819 | *F. acutatum* | KACC |
| K44820 | *F. avenaceum* | KACC |
| K42160 | *F. cerealis* | KACC |
| K42169 | *F. cerealis* | KACC |
| K42172 | *F. cerealis* | KACC |
| K42173 | *F. cerealis* | KACC |
| K44021 | *F. fujikuroi* | KACC |
| K44019 | *F. fujikuroi* | KACC |
| K44017 | *F. fujikuroi* | KACC |
| K44015 | *F. fujikuroi* | KACC |
| K44009 | *F. fujikuroi* | KACC |
| K44004 | *F. fujikuroi* | KACC |
| K42687 | *F. fujikuroi* | KACC |
| K42686 | *F. fujikuroi* | KACC |
| K42702 | *F. globosum* | KACC |
| K42701 | *F. globosum* | KACC |
| K41035 | *F. lateritium* | KACC |
| K42689 | *F. nygamai* | CBS |
| K42688 | *F. nygamai* | CBS |
| K42271 | *F. oxysporum* f. sp. asparagi | CBS |
| K42273 | *F. oxysporum* f. sp. chrysanthemi | CBS |
| K42274 | *F. oxysporum* f. sp. ciceris | CBS |
| K42276 | *F. oxysporum* f. sp. conglutinans | CBS |
| K42275 | *F. oxysporum* f. sp. glycines | CBS |
lesions (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. stilboideus*] 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 1. The nucleotide sequences of *TEF1* from the reference *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×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 1. Continued

| Isolate | Species | Source |
|---------|---------|--------|
| O1564   | *F. oxysporum* f. sp. *cubense* race 1 | FRC |
| O1565   | *F. oxysporum* f. sp. *cubense* race 1 | CBS |
| O1566   | *F. oxysporum* f. sp. *cubense* race 1 | KACC |
| O1567   | *F. oxysporum* f. sp. *cubense* race 2 | FRC |
| O1568   | *F. oxysporum* f. sp. *cubense* race 4 | CBS |
| O1955   | *F. oxysporum* f. sp. *cubense* | FRC |
| O1968   | *F. oxysporum* f. sp. *cubense* | FRC |
| O1090   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O1092   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O1097   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O1101   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O1102   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O1973   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O2022   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O2023   | *F. oxysporum* f. sp. *radicis-lycopersici* | FRC |
| O1140   | *F. oxysporum* var. *redolens* (*F. redolens*) | FRC |
| O1265   | *F. oxysporum* var. *redolens* | CBS |
| O1266   | *F. oxysporum* var. *redolens* | FRC |
| O1320   | *F. oxysporum* var. *redolens* | FRC |
| O1380   | *F. oxysporum* var. *redolens* | FRC |
| O1523   | *F. oxysporum* var. *redolens* | FRC |
| O1792   | *F. oxysporum* var. *redolens* | FRC |
| O1793   | *F. oxysporum* var. *redolens* | FRC |
| O1891   | *F. oxysporum* var. *redolens* | FRC |
| O1893   | *F. oxysporum* var. *redolens* | FRC |
| O1926   | *F. oxysporum* var. *redolens* | FRC |
| S0201   | *F. solani* f. sp. *cucurbitae* race 2 | FRC |
| S0202   | *F. solani* f. sp. *cucurbitae* race 2 | FRC |
| S023   | *F. solani* f. sp. *cucurbitae* race 2 | FRC |
| S0678   | *F. solani* f. sp. *cucurbitae* race 1 | FRC |
| S0688   | *F. solani* f. sp. *cucurbitae* race 1 | FRC |
| S0696   | *F. solani* f. sp. *cucurbitae* race 1 | FRC |

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

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

*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-alpha (*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×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
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 a ~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. stilboiodes* 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 examined 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.

**Table 3. Primers used to amplify DNA markers**

| Primer   | Sequence (5' → 3') | Amplified genes                  |
|----------|---------------------|----------------------------------|
| EF-1     | ATGGGTAAAGGA(A/G)GACAAGAC | *TEF1* (O’Donnell et al., 1998) |
| EF-2     | GGA(G/A)GTAACGATGG(C/G)ATCATGTT | *TUB1* (O’Donnell & Cigelnik, 1997) |
| tubT1    | AACATCGCTGAGATTTGAAGT | *RPB2* (Liu et al., 1999) |
| tubT22   | TCTGGATGTTGGAGAAATCC | *calM* (Mulè et al., 2004) |
| fRPB2-cF | ATGGG(G/C)TA(A/G)CAACG(G/C)ATGGG | |
| fRPB2-11aR | GC(A/G)TGGATCTT(A/G)TC(A/G)TC(C/G)ACC | |
| CLOX1    | CAGCAAAGCATCAGACCACTATAACTC | |
| CLOX2    | CTTGTCACTAACTGGAGCTTGTTACT | |

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. stilboiodes*, *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    | CGTGATTGGGAGGATGAGAGA      | forward primer for *F. solani* f. sp. *cucurbitae* (*Fsc*) race 1 |
| Fsc2    | ACGTGAGTGGAGACATGACGCGG    | forward primer for *Fsc* races 1&2, and *F. redolens*   |
| Tsolre1FM | ACATACCAATGAGCGGTGACATAGTA | forward primer for *Fsc* races 1&2, and other *F. solani* isolates |
| Fsolc1R | TGCCATTTGGGCGGCGGGGGGT     | reverse primer for *Fsc* race 1                         |
| Fsolc2R1| TTCACACAGACACTGACTCG        | reverse primer for *Fsc* race 2                         |
| Tsol1R  | CTGCTTATCTTTGGTCGGTGG      | reverse primer for *Fsc* races 1&2, and other *F. solani* isolates |
| Tredo1R | TTCGACTCGGCCGGTCCCA         | reverse primer for *F. redolens*                        |
| SmibolFM| GCAAAAAGCCTCTCGCCAC         | forward primer for both *F. stilboides* and *F. semitectum* |
| FstR1   | GACACCAATGATACCTTGGGC      | reverse primer for *F. stilboides*                      |
| Stibo1RM| GTGCTTCATTCGGAGAGTTTG      | reverse primer for *F. semitectum*                      |
| Semi1RM | AGGTTGAGAGATATCGCGG         | reverse primer for *F. semitectum*                      |

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

![Image](https://via.placeholder.com/150)

**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, S698, 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 Tso1R1 (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.

Fig. 4. Specific amplification of TEF1 regions from the isolates of F. stiboides and F. semitectum. (A) Upper panel: PCR amplification of a 578-bp fragment from genomic DNA of the F. stiboides isolates with the primers Smibo1FM and FstR1. Lanes 1-6: the F. stiboides 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. stiboides isolates with the primers Smibo1FM and StibolRM. 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 StibolRM. 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. stiboides 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. stiboides isolates (C319.73, C746.79, C101890, C101891, C101892, and C115624, respectively); 11-16: other Fusarium spp.

One primer (Smibo1FM) was a common forward primer for both species, whereas two reverse primers (FstI and Semi1RM) were specific for F. stiboides 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. stiboides and all of the F. semitectum var. majus, respectively (Fig. 4). The absence of the specific fragment found in F. stiboides C115624, which was identified as a member of the F. oxysporum complex clade instead of the F. stiboides clade in this study (Fig. 1), supports the species confirmation for five of the six F. stiboides isolates in the phylogenetic analysis. The only exception was that a DNA fragment was amplified from 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. stiboides (Geiser et al., 2005). To increase the reliability of the specific PCR detection developed for F. stiboides, 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 infraspecific 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 specialis 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 matrices should be screened. The PCR detection limit of fungal-specific DNA fragments from plant genomic DNA will be also determined.

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