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SHORT COMMUNICATION

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Detection method for *Fusarium torreyae* the canker pathogen of the critically endangered Florida torreya, *Torreya taxifolia*

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Abstract

Florida torreya (*Torreya taxifolia* Arn.) is an endangered conifer with a very limited range in the USA: two counties in Florida and one in Georgia, along the Apalachicola River. The species was once abundant in its small native range but suffered a major decline, ~99% loss, in the late 1950s to early 1960s that is thought to have been caused by a disease. Recently, a canker disease caused by *Fusarium torreyae* was identified as the primary cause of Florida torreya decline. Efforts to restore and preserve the species in situ and ex situ are hampered by lack of pathogen-free planting stock, and there exists an interest in methods to verify pathogen presence in seeds and seedlings prior to collection and transport for planting. This paper presents a new species-specific diagnostic method that enables detection of *F. torreyae* and may allow for conservation programmes to ensure germplasm is free of the pathogen prior to planting.

1 | INTRODUCTION

Florida torreya (*Torreya taxifolia* Arn.) is a critically endangered conifer listed by the U.S. Fish and Wildlife Service. The species has a limited native range consisting of ravines along the Apalachicola River, distributed in Liberty and Gadsden counties in Florida, USA, and in Decatur county Georgia (Smith et al., 2011). Historically, Florida torreya grew to 15–20 m and was relatively abundant, comprising about 14% of dominant (canopy) ravine trees, but experienced a dramatic decline from the late 1950s to early 1960s, suffering a ~99% loss in population size that was thought to be caused by a disease (Schwartz, Hermann, & van Mantgem, 2000). A survey by Smith et al. (2011) found 225 individuals with average heights from ground level of 78–263 cm and canker incidence of 71%–100% for the populations sampled. The cause of the canker disease was identified as *F. torreyae*, a previously undescribed species, and is currently a limiting factor in tree development (Aoki, Smith, Mount, Geiser, & O'Donnell, 2013; Smith et al., 2011). The current population size is unknown as no complete survey has been done since Hurricane Michael (category 5, 2018) which devastated the habitat of this species.

Many endangered species are in decline due to habitat loss, fragmentation, degradation or over-extraction. *Torreya taxifolia* is rather unique among endangered species as its habitat has remained relatively stable. In addition, it is not commercially used and is one of the few endangered plants recognized to be primarily threatened by disease (Schwartz et al., 2000). The pathogen that might have caused the rapid decline in the 1950s is not known, but the Florida torreya is currently being affected by a canker disease caused by *F. torreyae* and it is hypothesized that this disease might be responsible for the species' rapid decline (Aoki et al., 2013; Smith et al., 2011).

An ex situ conservation strategy was initiated with one cutting from each of 150 trees sampled and placed in four botanical gardens (Schwartz, 1993). Ex situ collections are ongoing (Smith et al., 2011), with the Atlanta Botanical Garden (Atlanta, GA) continuing to acquire new accessions and maintaining a large collection. Many of the botanical gardens were unable to maintain the collections, and most of the samples are currently found only at the Atlanta Botanical Garden. There is therefore a need to have ramets of these accessions at other locations to ensure their preservation. This is hampered by the necessity to ensure that the canker pathogen, *F. torreyae*, is not

found in seed or seedlings that will be sent to the new locations where that pathogen is currently not found. This study was undertaken to develop a rapid and inexpensive PCR-based method to identify *F. torreyae* from culture to help screen samples for the pathogen before propagules of *T. taxifolia* are moved to new locations.

2 | MATERIAL AND METHODS

2.1 | Primer design

Using the phylogenetic studies by Aoki et al. (2013) and Zhou et al. (2016) as guides, the genes encoding RNA polymerase II largest (RPB1) and second largest (RPB2) subunits were selected for development of a *F. torreyae*-specific PCR assay. The two recent studies (Aoki et al., 2013 and Zhou et al., 2016) were used to identify related taxa and sequences, supplemented with others as needed, then retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and aligned in Geneious 9.1.8 (https://www.geneious.com/). Areas that were unique to *F. torreyae* were identified, and primers were designed for these locations. An in silico specificity test using 114 sequences for both genes was performed by identifying all primer binding sites within both genes with no more than three mismatches in the binding region. Suitable primer pairs that were specific to *F. torreyae* were identified in both genes and synthesized for empirical testing.

2.2 | Specificity and sensitivity testing

The specificity of the PCR primers was then tested empirically using closely related species. The phylogenetic study by Zhou et al. (2016) was used to select taxa for the specificity test, which are listed in Table 1. The two closest relatives of *F. torreyae*, *F. zanthoxyii* and *F. continuum*, along with one taxon from each of the related species complexes and *F. aywerte*, representing a small but related clade, were chosen. *Fusarium dimerum* was also included because of its similarity in the RPB1 primer binding site. Isolates used in the study were obtained from the Agricultural Research Service Culture Collection (Peoria, IL), and additional information is available at https://nrrl.ncaur.usda.gov. Isolates were grown on PDA, DNA extracted following Dreaden et al. (2014), and the quality of the DNA confirmed by amplifying part of the rDNA internal transcribed spacer, ITS1-5.8s-ITS2, using general primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The DNA extracted from *F. torreyae* was quantified on a NanoDrop One and a serial dilution used to determine the lower detection limit of the assays.

TABLE 1 Taxa and isolates used for empirical specificity test

NRRL No.	Genus	Species
13384	*Fusarium*	*sublunatum*
13622	*Fusarium*	*lateritium*
20691	*Fusarium*	*dimerum*
22187	*Fusarium*	*sambucinum*
25331	*Fusarium*	*circinatum*
25410	*Fusarium*	*aywerte*
25473	*Fusarium*	*flocciferum*
66218	*Fusarium*	*continuum*
66285	*Fusarium*	*zanthoxyli*
54151	*Fusarium*	*torreyae*

PCR consisted of 1 μl PCR Buffer, 1 μl 2 mM deoxynucleotide triphosphates, 0.42 μl 50 mM $MgCl_2$, 0.08 μl Platinum Taq (Life Technologies), 0.4 μl 10 μM forward primer, 0.4 μl 10 μM reverse primer, 5.7 μl water and 1 μl DNA for 10 μl total PCR volume. Optimal primer annealing temperatures were determined by testing 65.4°C to 72°C. PCR thermocycling conditions were 94°C for 135 s followed by 40 cycles of 94°C for 20 s, 66°C for 10 s and 72°C for 30 s. DNA from *T. taxifolia*, samples 1,034 and 1,039, was obtained from the University of Florida Forest Pathology Lab to confirm the *F. torreyae* primers do not produce non-specific amplification when host DNA is present. PCR products were separated and visualized using 1.5% (m/v) agarose gels in 1x sodium borate buffer with GelRed nucleic acid stain (Biotium) co-loaded. The Ft_RPB2 primers and protocol were tested by the University of Florida Forest Pathology Lab using *F. torreyae* isolate PL510 to confirm the results were reproducible.

3 | RESULTS AND DISCUSSION

3.1 | RPB1

The *F. torreyae* RPB1 primers, Ft_RPB1_1250 GCAGTTGCCCCT CAACATT and Ft_RPB1_1362_R CCCTCACAGCAGGGATAACG amplified a 113-bp product with each primer having a single-bp mismatch with *F. zanthoxyli*. With a 68°C primer annealing temperature, only *F. torreyae* amplified. However, at these stringent conditions the limit of detection was only 0.1 ng/PCR. When lower primer annealing temperatures were tried, *F. zanthoxyli* amplified and the primers were not specific to *F. torreyae*. As a result, these primers were dropped from the study.

3.2 | RPB2

The *F. torreyae* RPB2 primers, Ft_RPB2_330 CCTGGTTAACTC AGTCTTGGATACG and Ft_RPB2_735_R ACGCTTCTCTTCTTCT GTGAGC amplified a 406-bp product (GenBank Accession MN908153). When tested against the 114 RPB sequences with a maximum of 3-bp mismatches allowed, only Ft_RPB2_735_R had a binding site in *F. zanthoxyli* with 2-bp mismatches. When the primers were compared to the entire GenBank nucleotide collection using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), matches were only found in *F. torreyae*. The optimum primer annealing temperature was 66°C and when used amplicons were only produced with

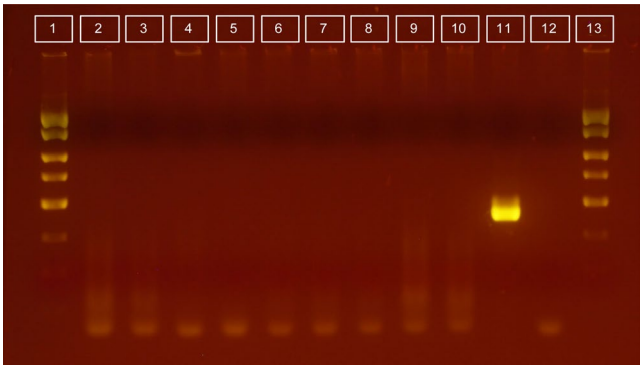

FIGURE 1 Agarose gel (1.5% m/v) showing the *Fusarium torreyae*-specific assay using RPB2 primers (Ft_RPB2_330 and Ft_RPB2_735_R). The specific 406-bp PCR product is only produced with *F. torreyae* (lane 11) and not from 9 closely related *Fusarium* spp. (lanes 2–10). Lanes 1 and 13 DNA marker (2 kb to 300 bp), 2 *F. sublunatum*, 3 *F. lateritium*, 4 *F. dimerum*, 5 *F. sambucinum*, 6 *F. circinatum*, 7 *F. aywerte*, 8 *F. flocciferum*, 9 *F. continuum*, 10 *F. zanthoxyli*, 11 *F. torreyae* and 12 TE Buffer

F. torreyae DNA, Figure 1. The lower detection limit was 0.01 ng of *F. torreyae* DNA per 10 µl PCR, data not shown.

For this assay, the quality of DNA from each sample must be confirmed before the results from the RPB2 *F. torreyae*-specific assay can be interpreted. We used ITS primers that worked well but their use requires an additional PCR per sample. An internal control was tested by utilizing a multiplex PCR with the RPB2 primers and *T. taxifolia* SSR primers (Ttax020F CAAGCATAAGTGATATCTGC and Ttax020R GAAGAAGAACAAGTAAAACG). With a 65°C primer annealing temperature, the RPB2 primers were specific, and the shorter SSR locus could be amplified and differentiated. However, the multiplex PCR with the SSR-based internal control increased the limit of detection to 0.1 ng for *F. torreyae* DNA per PCR. As a result, a separate PCR with ITS primers is the recommended method to confirm DNA quality prior to analysis and the multiplex PCR with internal control should not be utilized due to decreased sensitivity.

These primers were tested on 12 fungal cultures isolated from field samples collected from six *T. taxifolia*, two cankers per tree, yielding positive PCR assays for six isolates. Nucleotide sequence alignment (BLASTn) of positive PCRs against the NCBI database showed 100% identity to *F. torreyae*, supporting the specificity of these primers to identify the pathogen.

Current *T. taxifolia* conservation/restoration efforts include ex situ collections. While this will help maintain the genotypes, the movement of the plants is a pathway for also spreading the canker pathogen to new locations. The assay developed here can be used to screen *T. taxifolia* plants or seed before they are moved to new locations and thus limit the spread of the damaging canker pathogen that could affect other hosts in new environments (Trulock, 2013).

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