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Two novel *Fusarium* species that cause canker disease of prickly ash (*Zanthoxylum bungeanum*) in northern China form a novel clade with *Fusarium torreyae*

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**Abstract:** Canker disease of prickly ash (*Zanthoxylum bungeanum*) has caused a decline in the production of this economically important spice in northern China in the past 25 y. To identify the etiological agent, 38 fungal isolates were recovered from symptomatic tissues from trees in five provinces in China. These isolates were identified by conducting BLASTN queries of NCBI GenBank and phylogenetic analyses of DNA sequence data from the nuclear ribosomal internal transcribed spacer region (ITS rDNA), a portion of the translation elongation factor 1-α (*TEF1*) gene, and genes encoding RNA polymerase II largest (*RPB1*) and second largest (*RPB2*) subunits. Results of these analyses suggested that 30/38 isolates belonged to two novel fusaria most closely related to the Florida *torreyae* (*Torreya taxifolia* Arn.) pathogen, *Fusarium torreyae* in Florida and Georgia. These three canker-inducing tree pathogens form a novel clade within *Fusarium* here designated the *F. torreyae* species complex (FTOSC). BLASTN queries of GenBank also revealed that 5/38 isolates recovered from cankers represented an undescribed phylogenetic species within the *F. solani* species complex (FSSC) designated FSSC 6. Stem inoculations of three fusaria on *Z. bungeanum* resulted in consistent canker symptoms from which these three fusaria were recovered. The two novel fusaria, however, induced significantly larger lesions than FSSC 6. Herein, the two novel prickly ash pathogens are formally described as *F. zanthoxyli* and *F. continuum*.

**Key words:** *Fusarium continuum*, *F. zanthoxyli*, genealogical concordance, molecular phylogenetics, morphology, *RPB2*, *TEF1*

**INTRODUCTION**

Commonly known as prickly ash, *Zanthoxylum bungeanum* (Rutaceae) is an economically important tree species in dry, mountainous areas in several provinces in northern China. In the past two and one-half decades a canker disease has resulted in diminished production of prickly ash, a common peppery spice used in Asian cuisine derived from at least two species of *Zanthoxylum* including *Z. bungeanum*. The pericarp of this plant also is used in traditional Chinese medicine (Tang et al. 2014). Symptoms of the prickly ash disease include branch and stem cankers, dieback and occasional tree mortality. In initial studies conducted on canker disease of *Z. bungeanum* (hereafter abbreviated CDZB) in Shaanxi and Gansu provinces the putative pathogen isolated from cankered stems was identified as *Fusarium sambucium* Fuckel based on morphological data (Cao et al. 1992, 2010). Subsequently the CDZB isolates were re-identified as *F. lateritium* Nees (Xie 2012). These contrasting identifications highlight the need for the CDZB isolates to be characterized further with molecular systematic data. Phylogenetic species recognition based on genealogical concordance (Taylor et al. 2000) has made a significant impact on *Fusarium* systematics over the past two decades (Aoki et al. 2014). Portions of the translation elongation factor 1-α gene (*TEF1*, Geiser et al. 2004) and the largest (*RPB1*) and second largest (*RPB2*) subunits of RNA polymerase II genes have proven utility for inferring species limits and elucidating phylogenetic relationships within *Fusarium* (O’Donnell et al. 2013). Accordingly the present study was conducted to (i) collect and analyze multilocus DNA sequence data phylogenetically together with morphological data to identify the casual agents of *Fusarium* CDZB in the main prickly ash production areas of northern China, (ii) assess virulence of the fusaria on *Z. bungeanum* and (iii) formally describe two novel CDZB pathogens morphologically as *F. zanthoxyli* and *F. continuum*. 

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MATERIALS AND METHODS

Isolation of pathogenic fusaria.—Cankers on *Z. bungeanum* were collected 2010–2013 mainly in Shaanxi, Gansu and Shandong provinces but also in Shanxi and Hebei in China (FIG. 1). Thirty-eight *Fusarium* isolates (SUPPLEMENTARY TABLE I) were recovered from surface-sterilized stem tissues excised from the margins of cankers following the protocol of Smith et al. (2011). For long-term preservation all CDZB isolates were stored in 40% glycerol at −80°C in the laboratory of Zhi-Min Cao. In addition, two isolates of *F. zanthoxyli* and *F. continuum*, including the ex-type isolates, were deposited in the ARS Culture Collection (NRRL) and the CBS-KNAW Biodiversity Centre (see SUPPLEMENTARY TABLE I).

Herbarium specimens were deposited in the Mycological Herbarium of Forestry College, Northwest A&F University, Yangling, Shaanxi province, China (HMNWAFU).

DNA extraction, PCR amplification and sequencing.—Mycelium was cultured in potato dextrose broth (Liang et al. 2014) on a rotary shaker at 120 rpm for 7 d at 24°C. Mycelium was harvested over sterilized gauze, freeze-dried, and then total genomic DNA was extracted with a CTAB (hexadecyltrimethylammonium bromide) miniprep protocol (O’Donnell et al. 1998a). The nuclear ribosomal internal transcribed spacer region (ITS rDNA) and portions of *TEF1*, *RPB1* and *RPB2* genes were PCR-amplified and sequenced with primers published in the following: ITS rDNA (White et al. 1990), *TEF1* (O’Donnell et al. 1998b, 2008), *RPB1* (O’Donnell et al. 2010) and *RPB2* (Liu et al. 1999, Reeb et al. 2004). PCR reactions were conducted in a total volume of 50 μL that contained 2 μL of each primer (10 μM), 25 μL 2× *Taq* PCR MasterMix (Biosci Biotech Co., Hangzhou, China), 2 μL diluted (1:50) template DNA and 19 μL double-distilled water. After PCR amplification amplicons were sized by gel electrophoresis on 1.5% agarose gels that were run in 1× TAE buffer. Amplicons were purified and sequenced by Sangon Biotech Ltd, Shanghai, China.

Molecular phylogenetics.—Chromas 2 and Chromas Pro 1.7.5 (Technelysium Pty Ltd, Brisbane, Australia), respectively, were used to manually edit chromatograms and assemble the contigs. Contigs were aligned with MUSCLE (Edgar 2004) and the following two datasets were analyzed phylogenetically: (i) a 39-taxon *RPB1-RPB2* dataset to place the CDZB isolates within *Fusarium* (FIG. 2), and (ii) a 31-taxon three-gene dataset to assess the genealogical exclusivity of the two putatively novel CDZB pathogens, *F. zanthoxyli* and *F. continuum*. 

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**FIG. 1.** Collection sites in five Chinese provinces where *Fusarium zanthoxyli* (●) and *F. continuum* (▲) were isolated from *Zanthoxylum bungeanum*. Note that the range of these two pathogens overlaps in Fuping County, which is in central Shaanxi province.
FIG. 2. One of two most-parsimonious phylograms inferred from a combined $\textit{RPB1-RPB2}$ dataset that strongly supported the monophyly of seven species complexes within $\textit{Fusarium}$, including a novel clade of canker pathogens here designated the $\textit{F. torreyae}$ species complex. $\textit{Fusarium torreyae}$ is strongly supported as sister to the two novel $\textit{Zanthoxylum bungeanum}$ pathogens, $\textit{F. zanthoxyli}$ and $\textit{F. continuum}$. Canker disease of $\textit{Z. bungeanum}$ (CDZB) also can be induced by isolates of phylogenetic species $\textit{FSSC 6}$ in the $\textit{FSSC}$. Sequences of the $\textit{FSSC}$ were used to root the phylogram. Boldface identifies the 11 isolates from $\textit{Z. bungeanum}$ cankers included in the analysis. The number above internodes represents ML-BS value based on 1000 pseudoreplicates of the data. The MP-BS value is indicated only when it differed by $\geq 5\%$ the ML-BS value (ML-BS$\times$MP-BS). PIC = parsimony informative character, MPTs = most-parsimonious trees, CI = consistency index, RI = retention index.
*F. continuum* (Fig. 3). The individual and combined partitions were analyzed phylogenetically and clade support was assessed by maximum parsimony bootstrapping (MP-BS) in PAUP* 4.0b10 (Swofford 2003), and maximum likelihood (ML) in GARLI 2.01 on XSEDE (Zwickl 2006) on the CIPRES Science Gateway (http://www.phylo.org/portal2/login! input.action). The MP-BS analysis was conducted with equally weighted characters, a heuristic search employing 1000 random sequence additions, TBR branch-swapping, MaxTrees set at 5000 employing 1000 bootstrap replicates (Felsenstein 1985). ML bootstrapping was conducted employing the GTR + G + I model of molecular evolution, which was selected with JModelTest (Posada 2008). DNA sequence data generated in the present study were deposited in GenBank (Supplementary Table I), and alignments and trees were submitted to TreeBASE (accession number S17885, tree number Tr89189–Tr89193).

Pathogenicity of *Fusarium* to *Zanthoxylum bungeanum*.—To assess pathogenicity 38 isolates were inoculated onto eight 7 y old *Z. bungeanum* cultivar Doujiao trees in the nursery of the College of Forestry, Northwest A&F University. After a wound approximately 40 mm² was made with a pointed tweezer on 1 or 2 y old branches of pricky ash, it was inoculated with a 5 mm diam potato dextrose agar (PDA) plug containing mycelium from a 14 d old culture of one of the 38 isolates (Supplementary Table I), after which the inoculation site was wrapped with absorbent cotton dipped in sterile water and sealed tightly with a plastic film to keep humid. Every isolate was inoculated in two wounds on two different branches. Wounds inoculated with a sterile PDA plug served as the negative control. During the pathogenicity experiment the average temperature in the field was 20 C during daytime and 12 C at night. The experiment was terminated after 22 d at which time inoculated branches were removed from the trees and were taken to the lab where canker size was measured (Fig. 4). The average lesion dimension was measured and used in the ANOVA analysis with the LSD multiple comparison test employing IBM SPSS Statistics 19 (https://www14.software.ibm.com/). To complete Koch’s postulates, the inoculated isolates were re-isolated as described above and confirmed morphologically and molecularly by ITS sequence analysis.

Morphological characterization.—Fourteen isolates of *F. zanthoxyli* and five of *F. continuum* were used in the morphological study. Methods for determining phenotypic characters and mycelial growth rates followed Aoki et al. (2003, 2005). Isolates were grown at 20 C in 9 cm plastic Petri dishes on PDA and synthetic nutrient-poor agar (SNA) with or without placing a sterile 1 × 1 cm piece of filter paper on the SNA surface (Nirenberg and O’Donnell 1998) in the dark, under continuous fluorescent light (Panasonic YZ36RR6500K) or under daylight. Colony morphology, color, odor and growth rate were based on cultures grown on PDA (Fang 1998). All colors are given according to the Methylene Handbook of Color (Kornerup and Wanscher 1978). Mycelial growth rates were calculated as described in Aoki et al. (2003). Agar blocks 5 mm diam were cut from the margins of 2 wk old cultures on SNA and inoculated onto PDA. The cultures were incubated in the dark at eight temperatures 5–40 C at 5 C intervals. Cultures were examined after 1 and 5 d under a dissecting microscope, and colony margins were marked on the reverse side of the Petri dishes. Mean values of radial mycelial growth rate per day were calculated by measuring the distance from 16 points on the colony margin to the center. Measurements were repeated twice and averaged. All microscopic studies and measurements were made on isolates cultured on SNA. Measurements of 50 randomly selected conidia were taken based on the number of septa and cultural condition, and minimal and maximal sizes, arithmetic means and standard deviations (SD) were obtained. Conidia, conidiophores and chlamydo- spores produced on SNA were viewed and photographed by light microscopy (Olympus, CX31RTSF, Japan) with or without mounting them in water. Descriptive terms for conidia and conidiophore morphology followed Nirenberg and O’Donnell (1998).

RESULTS

DNA sequence-based identification of the isolates.—ITS rDNA, TEF1 and RPB2 nucleotide sequences of the 38 isolates recovered from *Z. bungeanum* cankers were used to query GenBank; TEF1 also was used to query FUSARIUM-ID (Geiser et al. 2004). Nucleotide BLASTN queries revealed that *Fusarium torreyae* was the best match for 30/38 isolates (Supplementary Table I) as follows: ITS rDNA = 97%, TEF1 = 87–91% and RPB2 = 95–96%. Sequences of 5/38 isolates that were nested within the FSSC (F201331–201135) revealed 99–100% identity to an unnamed phylogenetic species designated FSSC 6 (O’Donnell et al. 2008). The remaining three isolates were identified via BLASTN queries of GenBank, using the partial RPB2, as *F. acuminatum* (F201136, 100% identity to HM068334.1 F. acuminatum NRRL 54216), *Fusarium* sp. (F201237, 96% identity to JX171571.1 F. lateritium NRRL 13622) and an unnamed species within the *F. incarnatum-equiseti* species complex designated FIESC 1 (F201338, 100% identity to GQ505814.1 Fusicracid sp. FIESC 1, O’Donnell et al. 2009).

Molecular phylogenetics.—To place the two putatively novel isolates within *Fusarium*, we conducted a MP analysis of a 39-taxon RPB1–RPB2 dataset spanning the phylogenetic breadth of the genus that included sequences of five isolates of *F. zanthoxyli* and *F. continuum* (Fig. 2). MP analysis of the two-gene dataset outgroup-rooted on sequences of six members of the FSSC (Fig. 2) based on more inclusive analyses (O’Donnell et al. 2013), in which 1145/3368 characters were parsimony informative, found two most-parsimonious trees of 4486 steps (CI = 0.42, RI = 0.73) that differed only in the branching order of *F. illudens* C. Booth NRRL 22090 within the FSSC. The ML analysis produced a topologically similar tree (data not shown). ML and MP bootstrapping
FIG. 3. Maximum parsimony (MP) phylograms inferred from 31 aligned DNA sequences of the three fusaria comprising the FTOSC, which included data from ITS rDNA, TEF1, RPB2 and the combined three gene dataset. The phylograms were rooted on sequences of F. torreyae. Note that F. zanthoxyli and F. continuum are strongly supported as genealogical exclusive sister taxa by ML and MP (ML-BS, MP-BS) bootstrapping. PIC = parsimony informative character, MPT(s) = most-parsimonious tree(s), CI = consistency index, RI = retention index.
supported 27 and 28 nodes, respectively, at ≥70% (Fig. 2) and provided strong support for a novel clade (i.e., FTOSC) comprising *F. torreyae* and the two novel CDZB pathogens *F. zanthoxyli* and *F. continuum* whose monophyly (ML-BS/MP-BS = 100%) and sister group relationship were strongly supported (ML-BS/MP-BS = 92%). Although the three partitions we sequenced possessed different levels of phylogenetically informative characters (PIC) (Table I; ITS rDNA = 2.9%, *TEF1* = 17.1%, and *RPB2* = 8.9%), analyses of the individual ITS rDNA, *TEF1* and *RPB2* partitions and the combined three-gene dataset, using sequences of *F. torreyae* to root the phylogenies, strongly supported the genealogical exclusivity *F. zanthoxyli* and *F. continuum* (Fig. 3). Considerable allelic diversity was detected within both species, in that 20/24 *F. zanthoxyli* and all five *F. continuum* isolates possessed unique multilocus haplotypes. In addition, *F. zanthoxyli* isolates F201112 from Shaanxi and F201125 from Shanxi possessed highly divergent *TEF1* and *RPB2* alleles, respectively, (Fig. 3). *Fusarium continuum* isolates were collected from Shandong (n = 3) and Hebei (n = 1) and isolates of *F. zanthoxyli* were from Gansu (n = 10), Shaanxi (n = 14) and Shanxi (n = 1). However, *F. continuum* (F201030) and *F. zanthoxyli* (F201307) were sympatric in Fuping County, Shaanxi (Fig. 1). 

Pathogenicity of isolates to *Zanthoxylum bungeanum*.—Thirty-five of the 38 *Fusarium* isolates tested induced cankers (Figs. 5–7) and were recovered from the canker margins when the pathogenicity experiment was terminated after 22 d. Compared with isolates of *F. zanthoxyli* and *F. continuum*, the single isolate of *F. acuminatum* F201136, *Fusarium* sp. F201237 (*F. lateritium* species complex) and *Fusarium* sp. FIESC 1 F201338 (*F. incarnatum-equiseti* species complex) induced lesions on *Z bungeanum* cultivar Doujiao that were not significantly larger than the negative control (Fig. 8).

![Figs. 5–8. Canker symptoms exhibited by *Zanthoxylum bungeanum* cultivar Doujiao inoculated with (5) *Fusarium zanthoxyli* F201119, (6) *F. continuum* F201127 and (7) *Fusarium* sp. FSSC 6 F201131. 8. Negative control lacking a discernible canker.](image)

**Table I.** Tree statistics for individual partitions and combined dataset (see Fig. 3)

<table>
<thead>
<tr>
<th></th>
<th>MPTs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tree length</th>
<th>CI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>RF&lt;sup&gt;c&lt;/sup&gt;</th>
<th>bp&lt;sup&gt;d&lt;/sup&gt;</th>
<th>AUT&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PIC&lt;sup&gt;f&lt;/sup&gt;</th>
<th>PIC/bp&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS rDNA</td>
<td>1</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>548</td>
<td>2</td>
<td>16</td>
<td>0.029</td>
</tr>
<tr>
<td><em>TEF1</em></td>
<td>1</td>
<td>101</td>
<td>0.95</td>
<td>0.99</td>
<td>479</td>
<td>6</td>
<td>82</td>
<td>0.171</td>
</tr>
<tr>
<td><em>RPB2</em></td>
<td>3</td>
<td>181</td>
<td>0.96</td>
<td>0.99</td>
<td>1762</td>
<td>14</td>
<td>157</td>
<td>0.089</td>
</tr>
<tr>
<td>Combined</td>
<td>56</td>
<td>321</td>
<td>0.90</td>
<td>0.97</td>
<td>2789</td>
<td>22</td>
<td>255</td>
<td>0.091</td>
</tr>
</tbody>
</table>

<sup>a</sup> MPTs, most-parsimonious trees.
<sup>b</sup> CI, consistency index.
<sup>c</sup> RI, retention index.
<sup>d</sup> bp, base pairs.
<sup>e</sup> AUT, autapomorphy or a derived character unique to a particular taxon (i.e. not parsimony-informative).
<sup>f</sup> PIC, parsimony-informative or shared derived character.
<sup>g</sup> PIC/bp, parsimony-informative characters/bp.
Therefore we focused on results of the pathogenicity experiment obtained for isolates of *Fusarium zanthoxyli* (n = 25), *Fusarium continuum* (n = 5) and FSSC 6 (n = 5). Comparisons using Fisher’s LSD multiple comparison test revealed that *Fusarium zanthoxyli* and *Fusarium continuum* produced significantly larger lesions (mean = 1.24 ± 0.03 [SEM] and mean = 1.36 ± 0.05 [SEM], respectively) than the negative control (mean = 0.67 ± 0.01 [SEM]) (p < 0.01). Moreover, the five isolates of FSSC 6 induced significantly larger lesions (mean = 0.94 ± 0.03 [SEM]) than the negative control (p < 0.01). While lesions induced by *Fusarium zanthoxyli* and *Fusarium continuum* did not differ in size (p > 0.05), both were significantly larger than those produced by the five FSSC 6 isolates (p < 0.01), indicating that *Fusarium zanthoxyli* and *Fusarium continuum* were more virulent to *Zanthoxylum bungeanum* cultivar Doujiao.

**Taxonomy**

*Fusarium zanthoxyli* Z. Zhou, T. Aoki, K. O’Donnell & Z.M. Cao, sp. nov. Fggs. 9–31 MycoBank MB809716

**Typification:** CHINA. SHAANXI PROVINCE: Tongchuan city, Yaozhou district, Sunyuan town, isolated from stem tissue of diseased *Zanthoxylum bungeanum*., 4 Sep 2013, Xue Zhou Fyzs133-2013 (holotype HMNWAFU XZ-Fyzs133-20130408, a dried culture of F201311). Ex-type culture: F201311 = NRRL 66285 = CBS 140838.

**Etymology:** From Latin *zanthoxyli*, referring to the host, *Zanthoxylum bungeanum*.

Colonies on PDA with mycelial growth rates of 0.6–2.4 mm/d at 20 C in the dark. Colony margins mostly undulate, sometimes entire. Aerial mycelia on PDA generally sparsely to moderately formed, some developed abundantly, then loose to densely floccose, sometimes fritted, white (1A1), yellowish white (2–4A2), pinkish white to pastel red (7A2–4) in the dark, pastel red to reddish orange (7A4–6), pale red (7–9A3) under fluorescent or daylight, upon sporulation pale orange to deep orange (6A3–8). Pigmentation in the reverse pale yellow (2–4A3), pale orange to light orange (6A3–4) or pale red to pastel red (7A3–4) in the dark, light orange to orange (6A4–7), pastel red to reddish orange (7A5–7) under fluorescent or daylight. Dark blue sclerotial bodies sometimes present under daylight. Odor sweet, sometimes absent. Sporulation on SNA generally relatively scarce either directly on aerial mycelium, on substrate mycelium or in sporodochia. Sporodochia sometimes formed around the inoculum under fluorescent or daylight. Aerial conidiophores and conidia mostly absent or rarely formed on SNA. Aerial and sporodochial conidiophores and conidia not well differentiated. Aerial and sporodochial conidiophores, if present, generally densely to sparsely branched but sometimes unbranched, forming apical monophialides or sometimes intercalary phialides, up to 62.0 μm long and 2.5–6.5 μm wide. Phialides subcylindrical to ampulliform, often with a conspicuous collarate at the tip, up to 40.5 μm long and 2.5–5.0 μm wide. Aerial and sporodochial conidia morphologically indistinguishable, typically falcate, slightly curved or straight, dorsi-ventral, or sometimes fusiform, often widest in the upper half or less frequently at the midregion, tapering toward both ends, with an apical cell often rostrate, sometimes acute, and a basal foot cell indistinct to distinct with a conspicuous protrusion, (1–)3–5–(7–) septate; in darkness or under daylight, three-septate: 19.5–55.0 × 3.0–6.0 μm in total range, 30.7–42.5 × 3.7–4.5 μm av. (ex-type: 25.3–48.0 × 3.0–5.0 μm, 39.1 ± 7.3 ± 3.4 ± 0.5 μm av. and SD); five-septate: 41.0–76.0 × 3.7–6.5 μm, 54.3–68.5 × 4.7–5.8 μm av. (ex-type: 47.2–74.0 × 4.3–5.8 μm, 62.3 ± 5.9 ± 5.0 ± 0.3 μm av. and SD). Occasionally shorter, naviculate, ellipsoid to clavate conidia with acuate to pointed, rarely rounded apex and pointed to rounded base less frequently formed on SNA, (0–)1–2–(3–) septate, 7.5–28.0 × 2.0–4.0 μm if present. Chlamydoospores present or absent, oblong to subglobose, smooth to rough, thick-walled, intercalary or terminal, solitary, in pairs or catenate, 3.0–16.5 × 3.0–8.5 μm when present.

**Other isolates examined:** All from stem tissue of diseased *Zanthoxylum bungeanum*, CHINA. SHAANXI PROVINCE: Baoji city, Chencang district, Ershilipu, Sep 2011, Ning Xie Fgqs-2011, F201108; CHINA. GANSU PROVINCE: Hancheng city, Xuefeng town, Wang village, Aug 2011, Ning Xie Fhka-2011, F201108; CHINA. GANSU PROVINCE: Qinzhou district, Guanzi town, Sep 2011, Ning Xie Fgsy-2011, F201115; CHINA. GANSU PROVINCE: Gangu County, Ershilipu, Sep 2011, Ning Xie Fggs-2011, F201116; CHINA. GANSU PROVINCE: Gangu County, Daxiangshan town, Zhangjiaying village, Sep 2011, Ning Xie Fgsr-2011, F201117; CHINA. GANSU PROVINCE: Tianshui city, Qinzhou district, Guanzi town, Sep 2011, Ning Xie Fgfg-2011, F201119; CHINA. GANSU PROVINCE: Wen County, 25, 26, 29. Catenate chlamydoospores in hyphae and conidia. 27, 28, 30, 31. Short 1–3-septate, naviculate to clavate conidia with acute to rounded apex and pointed to rounded base. 9, 22 from F201119; 10, 11–16, 23, 30 from F201124; 17, 28 from F201311 (ex holotype); 18 from F201116; 19, 20, 26 from F201315; 21, 29 from F201108; 24 from F201218; 25 from F201307; 27 from F201322; 31 from F201117. Bars: 9–11, 18 = 50 μm, 12–17, 19–31 = 20 μm.
However, conidia in both species form long, slender sporodochial conidia. *F. zanthoxyli* distinguishes *Fusarium zanthoxyli* sporodochial conidiophores that are not well differentiated, typically falcate and gradually curved, with a rounded or truncate base. Aerial and sporodochial conidia morphologically indistinguishable, typically falcate and gradually curved, with a rounded or truncate base. Aerial and sporodochial conidiophores formed sparsely to abundantly, unbranched, sparsely or densely branched, forming intercalary or apical monophialides, up to 60.5 μm long and 2.5–6.0 μm wide. Phialides subcylindrical or ampuliform, often with a conspicuous collarette at the tip, up to 38.5 μm long and 1.5–4.5 μm wide. Aerial and sporodochial conidia morphologically indistinguishable, typically falcate and gradually curved, dorsiventral, or sometimes fusiform, mostly widest in the upper half, sometimes at the midregion, tapering and gradually curving toward both ends, with an apical cell mostly acuminate, sometimes pointed, and a basal foot cell indistinct to distinct, sometimes with a discernible protrusion; (1–)3–5–(6)-septate; in darkness three-septate: 16.0–48.0 × 3.0–5.5 μm, 34.5–38.9 × 4.3–4.7 μm av. (ex-type: 22.3–44.0 × 4.0–5.6 μm, 34.5 ± 5.1 × 4.7 ± 0.4 μm av. and SD); five-septate: 33.0–67.0 × 4.0–5.8 μm, 45.3–57.4 × 4.9–5.0 μm av. (ex-type: 33.0–53.1 × 4.1–5.5 μm, 45.3 ± 3.3 × 5.0 ± 0.2 μm av. and SD); On SNA sometimes shorter, ellipsoidal, naviculate to clavate conidia also formed, straight or curved, with a rounded, rarely pointed apex and a truncate or rounded base, 0–2–(3)-septate.

Conidiophores forming conidia. 46, 47. Falcate sporodochial conidia with acuminate apical cell and shorter conidia. 48, 49. Solitary, paired or catenate chlamydospores in conidia. 50–53. Short 0–3-septate, ellipsoidal, naviculate to clavate conidia with rounded to acute apex and rounded or truncate base. 32, 34, 38, 40, 41, 43, 44, 48, 49, 53 from F201127; 33, 35, 36, 47 from F201128; 37, 39 from F201129; 42, 45, 46, 51, 52 from F201030 (ex holotype); 50 from F201126. Bars: 32–35, 43, 44 = 50 μm; 36–42, 45–53 = 20 μm.
4.0–26.5 × 2.0–5.0 μm when present. Chlamydospores formed on hyphae or in conidia, subglobose to oblong, smooth to rough, thick-walled, intercalary or terminal, solitary, in pairs or catenate, 3.0–12.5 × 2.5–8.0 μm.


**Distribution:** Shandong, Hebei, and Shaanxi provinces, China.

**Notes:** *Fusarium continuum* can be differentiated from other members of the FTOSC by the production of shorter aerial and sporodochial conidia and rare production of 7–9-septate conidia, together with the undifferentiated aerial and sporodochial conidiophores. Of the three species within the FTOSC, conidia of *F. torreyae* are the longest, followed by *F. zanthoxyli* and *F. continuum*, when conidia with the same number of septa were compared. *Fusarium continuum* and *F. zanthoxyli* produce morphologically similar multisep-tate, falcate conidia that are usually widest in the upper half and only rarely at the midregion. By contrast those produced by *F. torreyae* are long, slender, gradually curved, and most frequently widest at the mid-region. Conidia produced by *F. continuum* can be distinguished from those of *F. zanthoxyli* in that the latter possess a rostrate apical cell and a conspicuous ventrally protruding basal cell. In addition, *F. continuum* also forms ellipsoidal, clavate to naviculate, straight or slightly curved conidia with a mostly rounded apex and rounded to truncated base on SNA. These two species also can be distinguished by differences in their radial mycelial growth rates. Average radial mycelial growth rates on PDA in the dark at eight temperatures 5–40 C were calculated for 14 isolates of *F. zanthoxyli* and five isolates of *F. continuum* and are summarized (Figs. 54, 55). Optimal temperature for mycelial growth was 25 C for all isolates of *F. zanthoxyli* and one isolate (F201126) of *F. continuum* and 30 C for 4/5 isolates of *F. continuum*. Mycelial growth at 25 C was 0.8–2.6 mm/d for *F. zanthoxyli* and 1.2–1.8 mm/d for *F. continuum*; growth rate at 30 C was 0.2–1.3 mm/d for *F. zanthoxyli* and 1.4–2.6 mm/d for *F. continuum*.

**DISCUSSION**

The primary findings of the present study include the discovery of a novel clade of canker-inducing fusaria, here designated as FTOSC, comprising *F. torreyae*, a pathogen of the critically endangered Florida torreya (*Torreya taxifolia*) in northern Florida and southwestern Georgia (Smith et al. 2011, Aoki et al. 2013) and two novel pathogens of prickly ash trees (*Zanthoxylum bungeanum*) in northern China that are described formally herein as *F. zanthoxyli* and *F. continuum*. Molecular clock estimates place the divergence of the FTOSC in the mid-Eocene ~ 40 Mya (O’Donnell et al. 2013), but it remains an open question whether this clade first evolved in the Old or New World. Furthermore, it remains to be determined whether *F. torreyae* is native to North America and restricted to *T. taxifolia*. Surveys for *F. torreyae* on *Torreya* endemic to China are warranted because it is the modern area of diversity of this genus (Li et al. 2001) and because the putative Asian origin of the CDZB pathogens could indicate that the most recent common ancestor of the FTOSC...
evolved in Asia. Additional surveys also are needed to characterize the host range and geographic distribution of the two novel CDZB pathogens. The available data suggests their ranges are distinct, with *F. zanthoxyli* distributed across three provinces from Gansu and Shaanxi in the northwest to Shanxi in the north of China, whereas *F. continuum* is mainly distributed in Shandong in the east of China. The two prickly ash pathogens, however, are sympatric in Fuping County of Shandong in the east of China. The two prickly ash pathospecies of *Z. bungeanum* for *F. zanthoxyli* that Z. bungeanum firmed to be pathogenic to *Z. bungeanum* indicates the importance (i.e. citrus).

Although a sexual cycle has not been found in the three FTOSC species in the laboratory, the high multilocus haplotype diversity we detected within *F. zanthoxyli* and *F. continuum* suggests that these pathogens may possess a heterothallic sexual reproductive mode. A similar finding in the soybean sudden death syndrome pathogen, *F. tucumaniae* T. Aoki et al., led to the discovery of a sexual cycle by conducting laboratory crosses under light and temperature conditions optimal for perithecial production (Covert et al. 2007). Knowledge of a sexual cycle in the CDZB pathogens has important implications for disease management and control because sexually reproducing pathogens are much more likely to overcome host resistance. In addition to the high SNP diversity that we detected within *F. zanthoxyli* it is worth mentioning that highly divergent *TEF1*, *RPB2* and ITS rDNA alleles were detected in isolates of *F. zanthoxyli* from Shaanxi, Shanxi and Gansu, respectively. Of interest, the ITS rDNA of F201125 from Shanxi differed at only two nucleotide positions from that of *F. torreyae*, suggesting possible hybridization between *F. zanthoxyli* and a *F. torreyae*-like species.

In the present study we characterized a novel plant disease designated Canker disease of *Zanthoxylum bungeanum* (CDZB) and completed Koch’s postulates. Lesion sizes caused by the three most frequently isolated *Fusarium* species recovered from prickly ash trees showing symptoms of CDZB were used as a proxy for their virulence on this host. Results of the pathogenicity experiment revealed that isolates of the two novel CDZB-associated *Fusarium* species exhibited higher virulence than that of FSSC 6 and single isolates of three other *Fusarium* species. This finding establishes that *F. zanthoxyli* and *F. continuum* are the major pathogenic *Fusarium* species on *Z. bungeanum*. Isolates of *F. zanthoxyli* and *F. continuum* also were confirmed to be pathogenic to *Z. bungeanum* cultivars Dahongpao (including Fengjiao and Hancheng-Dahongpao), Youjiao and Mijiao (Xie 2012). Longer-term inoculation studies are needed to help elucidate whether the causal agents can ultimately kill trees under more field based conditions. It is also of interest to test these isolates against other members of the Rutaceae, especially those that are economically important (i.e. citrus).

Several species of *Fusarium* are capable of causing cankers on woody plants, and mixed infections frequently occur. For example, a particular FSSC species occasionally was found co-occurring with *F. torreyae* in cankers on Florida torreya (*Torreya taxifolia*) (Smith et al. 2011). Although both species could induce cankers, *F. torreyae* is considered to be the primary pathogen due to increased virulence and consistent isolation from a large number of cankers. By contrast the available data indicates the FSSC taxon should be regarded as an opportunistic (J. Smith pers comm). In addition, *F. solani* f. sp. *xanthoxyli*, a well-known fungus in Japan within the FSSC has been reported to cause trunk-blight of a closely related tree species, *Zanthoxylum piperatum* (L.) DC. under the name of *Neotria elegans* Yamamoto & Maeda (Yamamoto et al. 1957, Matuo and Snyder 1961, Sakurai and Matuo 1961). However, this fungus was shown to represent an undescribed phylogenetic species of *Fusarium* designated FSSC 22 (O’Donnell et al. 2008), which is morphologically different from the CDZB-associated *Fusarium* species from China. Although multiple species frequently are isolated from a host in disease etiology studies, pathogenicity and virulence testing are essential to complete Koch’s postulates, as is identification of the fungal species most frequently isolated from symptomatic tissues.

In addition to *Fusarium* species other microbes have been reported to induce cankers on *Z. bungeanum*, for example, *Phytophthora* spp. (Xie et al. 2013). Because these cankers are typical of those caused by other *Fusarium* spp. (i.e. FSSC 6 and *F. torreyae*) it is possible that two different stem diseases have been confused on this host. Also a jewel beetle (*Agrilus* sp.) frequently colonizes wounds resulting from CDZB (Li et al. 1988), causing further damage to this host. Whether the wounds caused by this wood-boring beetle serve as infection courts for these pathogens warrants further study.

The 38 fusaria isolated from *Zanthoxylum bungeanum* cankers in the present study were identified and placed in *Fusarium* by a two-step process that first involved BLASTN queries of NCBI GenBank and Fusarium-ID (Geiser et al. 2004), using a partial *TEF1* sequence as the query, followed by phylogenetic analyses of a *RPB1-RPB2* dataset that spanned the phylogenetic breadth of *Fusarium* (O’Donnell et al. 2013). Results of both analyses indicated that the two primary CDZB pathogens were most closely related to *F. torreyae* (Smith et al. 2011, Aoki et al. 2013). Morphological species recognition is challenging for nonspecialists in *Fusarium* (Gerlach and Nirenberg 1982, Nelson et al. 1983, Leslie and Summerell 2006), as evidenced by the phenotypic identification of *F. torreyae* as *F. lateritium* (El Gholl 1985) and the CDZB pathogens as *F.*
sambucinum (Cao et al. 1992, 2010) and F. lateritium (Xie et al. 2012), although Fusarium isolates from wooden substrates often have been identified as F. lateritium without detailed morphological analyses. Fusarium torreyae can be distinguished from F. zanthoxyli and F. continuum by its host, Florida torreya, and phenotypically by the frequent production of seven-septate sporodochial conidia and the absence of aerial conidia. In contrast F. continuum produces abundant conidia that are shorter than F. torreyae, rarely produces seven-septate sporodochial conidia and occasionally produces dark blue sclerotia. Fusarium zanthoxyli occasionally produces aerial conidia and falcate seven-septate sporodochial conidia that are shorter than F. lateritium—3-septate conidia. Because the tree space surrounding the F. torreyae, F. lateritium and F. buharicum species complexes contains approximately 20 undescribed species lineages (O’Donnell unpubl), which were resolved by phylogenetic species recognition based on genealogical concordance (Taylor et al. 2000), we advocate employing multilocus DNA sequence data for species identification and recognition within Fusarium coupled with detailed phenotypic analyses including morphology. Through this approach we discovered a novel clade of tree canker-inducing pathogens that includes the prickly ash pathogens F. zanthoxyli and F. continuum in China and the Florida torreya pathogen, F. torreyae in southeastern United States.

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