

Short title: *Ceratocystis tiliae* on *Tilia americana*

Ceratocystis tiliae sp. nov., a wound pathogen on *Tilia americana*

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Abstract: Species in the North American clade (NAC) of the *Ceratocystis fimbriata* complex are mostly weak pathogens that infect native tree hosts through fresh wounds. Isolations from discolored tissue of wounded *Tilia americana* (basswood) in Iowa and Nebraska yielded a *Ceratocystis* species that was similar to but distinct from isolates of *C. variospora* from other hosts. Sequences of 28S rDNA showed that isolates from basswood did not differ from *C. variospora*, but there were minor differences in ITS rDNA sequences. The DNA sequences of a portion of the Cerato-platanin gene and *TEF1* α showed the basswood fungus to be a unique lineage. Cross inoculations in two experiments showed that the basswood isolates and *C. variospora* isolates from *Quercus* spp. were most aggressive to their respective hosts. Isolates

from basswood grew slower and were less pigmented than *C. variospora* isolates from *Quercus* spp. The basswood fungus thus is distinguished from *C. variospora* based on phylogenetic analyses and phenotype and is herein described as *C. tiliae* sp. nov.

Key words: *Ceratocystis fimbriata*, *C. variospora*, ceratocystis wilt, genetic variability

INTRODUCTION

Several host-specialized but otherwise phenotypically indistinguishable species in the *Ceratocystis fimbriata* Ellis & Halst. complex have been named in recent years (Engelbrecht and Harrington 2005, Johnson et al. 2005, de Beer et al. 2014, Harrington et al. 2014). Members of the *C. fimbriata* complex are wound pathogens on root crops and may cause wilting and cankering on a wide variety of woody hosts (Harrington 2013). In North America woody hosts include *Quercus* and *Prunus* spp. (oaks, almonds and other stone fruits, by *C. variospora* R.W. Davidson), *Populus* spp. (aspen and other poplars, by *C. harringtonii* Z.W. de Beer & M.J. Wingfl.), *Carya* spp. (hickory, by *C. smalleyi* J.A. Johnson & T.C. Harrin. and *C. caryae* J.A. Johnson & T.C. Harrin.), and *Platanus* spp. (sycamore or plane tree, by *C. platani* (Walter) Engelbrecht & T.C. Harrin., a member of the Latin American Clade of the complex) (Engelbrecht and Harrington 2005, Johnson et al. 2005). Other recognized species in the Latin American Clade include *C. cacaofunesta* Engelbrecht & T.C. Harrin., which causes Ceratocystis wilt on *Theobroma* spp. (Engelbrecht and Harrington 2005) and *C. fimbriata*, strains of which cause rot of corms of *Colocasia esculenta* (taro) and other aroids as well as storage roots of *Ipomoea batatas* (sweet potato) worldwide, including eastern USA (Engelbrecht and Harrington 2005, Thorpe et al. 2005).

Limited morphological variation has made delimitation of species in the *C. fimbriata* complex difficult, and there appears to be many cryptic species awaiting formal description.

However description of new species based solely on variation in the ITS rDNA region has been problematic (Harrington et al. 2014, Fourie et al. 2015). Oliveira et al. (2015) contrasted different species recognition concepts for delimitation of species in the complex and recommended the traditional phylogenetic species concept, which requires a diagnosable phenotype to delimit phylogenetic lineages as species (Harrington and Rizzo 1999).

Johnson et al. (2005) revised the taxonomy of the North American Clade (NAC) of the *C. fimbriata* complex, resurrecting the name *C. variospora* and describing three new species: *C. populicola* J.A. Johnson & T.C. Harrin., *C. caryae* and *C. smalleyi*. *Ceratocystis populicola* later was found to be a homonym of *C. populicola* Olchow. & Reid, leading to a new basionym, *C. harringtonii* (de Beer et al. 2013). Several phylogenetic lineages within *C. variospora* were considered to be potential new species, including one isolated from wounds on American basswood, *Tilia americana*. Isolates from wounds on basswood were not interfertile with other strains of *C. variospora* from *Quercus* and *Prunus*, and their ITS rDNA sequences differed slightly.

Isolations from discoloration surrounding wounds on basswood trees in Iowa and Nebraska yielded isolates morphologically similar to but distinct from *C. variospora* and with unique ITS rDNA sequences, indicating that the basswood fungus was a unique lineage within the NAC that may be specialized to colonize wounds of this host. The aim of this study was to further determine if basswood strains are phylogenetically and phenotypically distinct from other members of the NAC and warrant separate species recognition.

MATERIALS AND METHODS

Collection of isolates.—Isolates were obtained from four living *Tilia americana* trees at three locations. The tree at the Ogden, Iowa, site was artificially wounded, and isolates of the fungus (C1954, C1959) were recovered from the edge of the wounded tissue (Johnson et al. 2005). A small-diameter tree in Ames, Iowa, was damaged from a larger

fallen tree, and the fungus was recovered (C2131) from the wounded area. A row of large, planted trees with substantial branch dieback was examined in Omaha, Nebraska. The trees had suffered storm damage several years earlier, and many of the major branches had sapwood discoloration coming down from the broken branch tops. Isolation attempts from two of the trees yielded six isolates (C2525 and C2623 from tree 1 and C2620, C2621, C2622 and C2624 from tree 2). In most cases the fungus was baited from the wounded area of discolored sapwood tissue with the carrot sandwich technique (Moller and deVay 1968). Ascospore masses from perithecia formed on the carrot disks were transferred to agar media for purification and storage. Only one isolate from each tree was used in phylogenetic analyses.

DNA extraction and sequencing.—Isolates were grown on MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) for 10 d at room temperature (approximately 23 C), and DNA was extracted from the superficial growth and sporulation using PrepMan™ Ultra (Applied Biosystems, Foster City, California).

The ITS rDNA sequences were generated with primers ITS1-F and ITS4 following the procedures of Johnson et al. (2005). The D1/D2 domain of the 28S (LSU) rDNA gene region was amplified with the primers LROR (5′-ACCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTGAGGGAACTTCG-3′) and sequenced with primers LROR and LR3 (5′-CCGTGTTTCAAGACGGG-3′). The thermo-cycler settings were 85 C for 120 s, 95 C for 95 s, 36 cycles of 58 C for 60 s, 72 C for 80 s and 95 C for 70 s, followed by 52 C for 60 s and 72 C for 15 min. A 500 bp fragment of the Cerato-platanin gene region (Pazzagli et al. 1999, Fontana et al. 2008, Chen et al. 2013) was amplified and sequenced with newly developed primers for the *C. fimbriata* complex based on the original sequence from *C. platanii*: CP-2F (5′-TCCTACCCATGATTGCCAGC-3′) and CP-1R (5′-ACAACAGCGTACTGCCTTCAT-3′), with thermo-cycler settings as in the PCR for 28S rDNA. For *TEF1α*, the primers EFCF1 (5′-AGTGCGGTGGTATCGACAAG-3′) and EFCF6 (5′-CATGTCACGGACGGCGAAAC-3′) were used to amplify a product of about 1600 bp that included portions of three exons and two introns (Harrington 2009, Oliveira et al. 2015). Additional primers EFCF2 (5′-TGCTCAACGGGTCTGGCCAT-3′) and EFCF3 (5′-ATGGCCAGACCCGTGAGCA-3′) also were used for sequencing. Thermo-cycler settings for amplifying the *TEF1α* region included an initial denaturation at 85 C for 120 s followed by 94 C for 95 s, with 36 cycles of 60 C for 60 s, 72 C for 90 s and 94 C for 35 s, followed by final extension of 60 C for 60 s and 72 C for 15 min.

Phylogenetic analyses.—Isolate C1476 (ICMP 8579) of *C. fimbriata* from sweet potato in Papua New Guinea was used as outgroup taxon for 28S rDNA, *TEF1α* and Cerato-platanin analyses because it is a member of the Latin

American Clade. Sequences of each of the three datasets were manually aligned, but some ambiguity was found in the alignment of the *TEF1 α* introns, and the intron regions therefore were eliminated from the analysis. Sequences of the three-gene regions were analyzed separately and a combined dataset was submitted to a partition homogeneity test (PHT) with PAUP 4.0b10 (Swofford 2002) to determine whether the datasets could be combined.

For maximum parsimony analysis (PAUP 4.0b10), gaps were treated as a fifth base, all characters had equal weight and the heuristic searches used simple stepwise addition and tree-bisection-reconnection. Bootstrap values were calculated with 1000 replications. For Bayesian analyses, the best model of nucleotide substitution for each gene was selected according to Akaike information criterion (AIC) using MrModeltest 2.3 (Nylander, 2004). Multigene Bayesian inference (BI) was conducted for the aligned dataset of the sequences of 19 isolates for 28S rDNA and for sequences of 34 isolates for the *TEF1 α* and Cerato-platanin datasets. The analyses were conducted with the software MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003) using the algorithm of Markov chain Monte Carlo (MCMC) with two sets of four chains (one cold and three heated) and the stoprule option, stopping the analysis at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 1000, and the first 25 percent of trees were removed. Phylogenetic trees were viewed and edited in Fig Tree 1.3.1.

(<http://tree.bio.ac.uk/software>).

Pathogenicity tests.—Host specialization was tested in two cross inoculation experiments with seedlings of two hosts, *T. americana* and *Quercus macrocarpa* (bur oak), three isolates (C1954, C2131, C2622) from *T. americana*, and three isolates of *C. variospora* (C1837, C1846, C1964) from wounds on *Quercus* spp. (*Q. rubra*, *Q. robur*, *Q. macrocarpa* respectively) in Iowa. For each of the two experiments, seven inoculation treatments, consisting of three isolates from each of the two hosts and a control, were applied to each host with three replicates (seedlings) in a random design. Dormant 12 mo old bareroot seedlings of basswood and bur oak were planted in 15 cm pots in potting medium amended with Osmocote slow-release fertilizer in a greenhouse and inoculated 6 wk after bud break. A self-fertile, single-ascospore strain of each isolate was transferred onto MYEA and grown at room temperature (≈ 23 C) for 10 d. The plants were wounded by making a 3-mm-deep, downward-slanting cut from the outer bark into the wood with a sterile scalpel at 3 cm above ground. Agar disks (5 mm diam) with mycelium and spores were placed in the wound, and the inoculation site was wrapped with Parafilm. After 60 d or at the time of death, each stem was sectioned vertically and the length of xylem discoloration above and below the point of inoculation was measured.

Morphological characterization.—Four representative isolates were grown on MYEA (2% malt extract, 0.2% yeast extract, 2% agar) for 10 d at room temperature (approximately 23 C). Measurements of conidia and conidiophores were made after 4–7 d, perithecia and ascospores were measured after 7–10 d, and aleurioconidia were measured after 7–20 d. Material was mounted in lactophenol/cotton blue and observed with Nomarsky interference microscopy. For most structures 10 observations were recorded per isolate; when measuring conidia; however 20 conidia were measured per isolate. Some structures were rare or hard to locate in a few isolates, and fewer observations were made. Colony pigmentation was compared to the color chart of Rayner (1970).

The growth rate (colony diam at 7 d) of the isolates from *T. americana* (C1954, C2131, C2622) were compared to isolates of *C. variospora* from *Quercus* spp. (C1837, C1846, C1964). Three replicate plates of each isolate were grown on MYEA at 25 C.

RESULTS

Phylogenetic analyses.—Harrington et al. (2014) and Fourie et al. (2015) indicated that the ITS rDNA region is problematic for phylogenetic analyses of the *C. fimbriata* complex, and Johnson et al. (2005) reported that *Tilia* isolates and other isolates of *C. variospora* had similar ITS rDNA sequences. In the present analysis the ITS rDNA sequences of four *Tilia* isolates (C1954, C1959, C2131, C2622) differed from each other only in the number of Ts in long poly-T stretches in the middle of the ITS1 region and near the end of ITS2. In comparisons with the ITS rDNA sequences of representative isolates of *C. variospora*, the sequences of the *Tilia* isolates differed in the number of Ts in four poly-T regions. This suggested that the ITS rDNA region was diagnostic for separating the *Tilia* isolates from *C. variospora*, but there did not appear to be a discernable phylogenetic signal.

The partition homogeneity test conducted for the three gene regions resulted in $P = 0.01$, which is considered low (Cunningham 1997). Therefore phylogenetic analyses were conducted for each gene region separately.

An alignment of 593 characters of 28S rDNA showed little variation among the 19 isolates analyzed, with 552 characters constant and only nine parsimony-informative characters. Two sequences were identified among the three analyzed isolates from *T. americana*, but these sequences were not distinguishable from those of some isolates of *C. variospora*, except for isolate C2131 (FIG. 1). Parsimony analysis resulted in one most parsimonious trees of 29 steps (FIG. 1) with homoplasy index (HI) = 0.1379, consistency index (CI) = 0.8621, rescaled consistency (RC) = 0.7871 and retention index (RI) = 0.9130. The evolution model GTR + I + G for 28S rDNA region was selected and incorporated into the Bayesian analysis, and the posterior probability values are indicated on the MP tree (FIG. 1)

The aligned sequences of 36 isolates for *TEF1* α consisted of 1410 characters, 1304 of which were constant and 67 were parsimony informative. Parsimony analysis resulted in 60 most parsimonious (MP) trees of 146 steps (FIG. 2), with HI = 0.1918, CI = 0.8082, RC = 0.7726 and RI = 0.9560. The evolution model HKY + G for *TEF1* α region was selected and incorporated into the Bayesian analysis. Only one *TEF1* α sequence was identified among the three *T. americana* isolates tested, and this sequence differed from those of all tested isolates of *C. variospora*.

Aligned sequences of the Cerato-platanin gene for 46 isolates resulted in an alignment of 502 characters, 324 of which were constant and 94 were parsimony informative. Parsimony analysis resulted in three most parsimonious trees of 222 steps (FIG. 3), with HI = 0.1081, CI = 0.8919, RC = 0.8726 and RI = 0.9784. The evolution model HKY + I + G for Cerato-platanin region was selected and incorporated into the Bayesian analysis. The three *T. americana* isolates tested had the same Cerato-platanin sequence, and this sequence differed from those of all the tested isolates of *C. variospora*.

Pathogenicity tests.—All inoculated plants showed xylem discoloration by the end of each of the two experiments (FIG. 4). The controls remained asymptomatic and had only a trace of xylem discoloration at the inoculation point. After evaluation of xylem discoloration, the inoculated pathogen was re-isolated from each of the seedlings, but no fungus was recovered from the controls.

In both experiments isolates from *T. americana* and *Quercus* spp. caused greater xylem discoloration in their respective hosts than in the other host (FIG. 5). The three *T. americana* seedlings inoculated with *T. americana* isolate C1954 died in the first experiment, and two of the three *T. americana* seedlings inoculated with *T. americana* isolate C2131 died in the second experiment. No other seedlings died.

The ANOVA showed significant variation in the length of xylem discoloration among the six isolates and between the two inoculated host species, and there was significant isolate \times host interaction in each experiment ($F = 13.38, P < 0.0001, F = 14.59, P < 0.0001$ respectively), indicating host specialization. The ANOVA showed a significant difference between the two experiments, and they were not combined in the analyses (FIG. 4) because variances of the two experiments were not homogeneous.

Morphological characterization.—Isolates obtained from *T. americana* were distinguished from *C. variospora* isolates from *Quercus* spp. in the pigmentation of their mycelia and extent of linear growth at 7 or 10 d on MYEA (FIG. 4). *Ceratocystis variospora* isolates from *Quercus* spp. were more darkly pigmented, tending to dark brown, while isolates from *T. americana* were initially whitish, then turned to pale brown-gray. The extent of linear growth (colony diam at 7 d) of the isolates from *T. americana* (C1954 = 3.8 cm, C2131 = 3.6 cm, and C2622 = 4.3 cm) was less than that of isolates of *C. variospora* from *Quercus* spp. (C1837 = 5.2 cm, C1846 = 5.3 cm, C1964 = 5.4 cm).

TAXONOMY

Ceratocystis tiliae L.S.S. Oliveira, D. McNew & T.C. Harrin., sp. nov. (FIG. 6A–F)

Mycobank MB810406

Typification: USA. IOWA. Ogden, dried culture isolated from *Tilia americana*, Jun 2002, J.A. Johnson (**holotype** BPI: 892985). Ex-type strains C1954 (=CBS 137354).

Etymology: *tiliae* (L), on *Tilia*.

Cultures on malt yeast-extract agar white, turning to pale brown-gray after 7–10 d at 25 C, undersurface of agar turning dark, especially under areas where perithecia were produced. Colony diam 35 mm at 7 d. Odor sweet, with banana scent. Perithecia with bases superficial to partially immersed, bases black or rarely dark brown, globose, 175–350 µm diam, unornamented or with undifferentiated hyphae, possessing a collar 50–100 µm wide at the base of the neck. Necks black or rarely dark brown, slender, 425–915 µm long, 25–45 µm wide at base and 15–35 µm at the tip. Ostiolar hyphae hyaline, 40–90 µm long, divergent. Asci not seen. Ascospores 5.0–6.0 × 4.0–4.5 µm with outer cell wall forming a hat-shaped brim. Conidiophores of two types: one flask-shaped, hyaline to pale brown, septate, 80–160(330) µm long, with phialides

25–50(–120) μm long, 4.0–5.5 μm wide at base tapering to 3.5–4.5 μm at the aperture; producing chains of hyaline, cylindrical conidia 15–40 \times 3.0–5.5 μm ; other conidiophores rarely found, shorter, 50–90 μm long, not tapering, phialides 25–50 μm long, 4.5–5.5 μm wide at base and often flared at aperture, 4.0–6.0 μm , producing chains of doliform conidia, hyaline, 6.5–9.0 \times 4.5–6.0 μm . Aleurioconidia produced singly or in chains from simple conidiophores, pale brown to dark brown, ovoid or obpyriform, smooth, 7.5–12.5 \times 8.0–11.5 μm .

Additional cultures examined: USA. IOWA. Ames, from *Tilia americana*, Aug 2004, T. C. Harrington, isolate C2131 (= CBS 137355, BPI 892984). NEBRASKA. Omaha, from *Tilia americana*, Sep 2009, T. C. Harrington, isolate C2622 (= CBS 137356).

DISCUSSION

The 28S rDNA sequence of isolates of *C. tiliae* is the same as that of some isolates of *C. variospora*, but the ITS rDNA of the *C. tiliae* isolates differed, and analyses of *TEF1 α* and Cerato-platanin DNA sequences showed that isolates of *C. tiliae* form a distinct phylogenetic lineage. In Johnson et al. (2005) isolates from *T. americana* had ITS sequences and allozymes similar to other members of the *C. variospora* group, but intersterility tests showed that isolates from *T. americana* were able to mate only with themselves and not with *Prunus* or *Quercus* isolates of *C. variospora*, suggesting that *C. tiliae* is a distinct biological species. It is now clear that *C. tiliae* is phenotypically distinct from *Quercus* isolates of *C. variospora* because the mycelium is less pigmented and it grows slower than *Quercus* isolates, and *C. tiliae* colonizes wounds of living *T. americana* more aggressively. Although isolates of *C. tiliae* were aggressive in inoculation tests and the fungus has been recovered from the sapwood below dying branches of *T. americana*, it is unclear whether *C. tiliae* kills branches or stems of *T. americana* in nature.

Johnson et al. (2005) suggested that there were likely cryptic species within *C. variospora*, but they were unable to characterize further species in the NAC using the

phylogenetic species concept due to the lack of diagnostic, phenotypic characters (Harrington and Rizzo 1999). Our more detailed phylogenetic and phenotypic characterizations clearly distinguish *C. tiliae*, but this recognition leaves *C. variospora* as a paraphyletic taxon, and other cryptic species are likely to be found in this group.

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LEGENDS

FIG. 1. A single most parsimonious tree of representatives of the North American Clade (NAC) of the *Ceratocystis fimbriata* complex based on sequences of 28S rDNA. Bootstrap values greater than 60%/posterior probability values greater than 0.9 are indicated on appropriate branches. Isolate numbers are followed by host genus, country and state. Isolate C1954 (= CBS 137354) is the ex-holotype culture. The tree was rooted to *C. fimbriata* in the Latin American Clade (C1476). Bar indicates the number of base changes per site.

FIG. 2. One of 60 most parsimonious trees of representatives of the North American Clade (NAC) of the *Ceratocystis fimbriata* complex based on sequences of *TEF1α*. Bootstrap values greater than 60%/posterior probability values greater than 0.8 are indicated on appropriate branches. Isolate numbers are followed by host genus, country and state. The tree was rooted to *C. fimbriata* in the Latin American Clade (C1476). Bar indicates the number of base changes per site.

FIG. 3. One of three most parsimonious trees of representatives of the North American Clade (NAC) of the *Ceratocystis fimbriata* complex based on sequences of Cerato-platanin. Bootstrap values greater than 70%/posterior probability values greater than 0.8 are indicated on appropriate branches. Isolate numbers are followed by host genus, country and state. Isolate C1954 (= CBS 137354) is the ex-holotype culture. The tree was rooted to *C. fimbriata* in the Latin American Clade (C1476). Bar indicates the number of base changes per site.

FIG. 4. Pathogenicity and colony characteristics of isolates of *Ceratocystis tiliae*. A. Wilting of a *Tilia americana* seedling (left) inoculated with a *C. tiliae* isolate (C1954 = CBS 137354) and a control seedling (right). B. Xylem discoloration caused by *C. tiliae* in a *T. americana* seedling. C. Difference in the extent of radial growth and pigmentation between *C. tiliae* isolate C1954 (left) and *C. variospora* isolate C1837 (right) after 10 d on malt yeast extract agar.

FIG. 5. Average length (cm) and standard deviation of xylem discoloration in *Quercus macrocarpa* (bur oak) and *Tilia americana* (basswood) seedlings wound inoculated with isolates of *Ceratocystis variospora* and *C. tiliae* obtained from *Quercus* spp. and *T. americana* respectively. Bars with the same letter are not significantly different from each other using Tukey's test ($P < 0.05$).

FIG. 6. Morphological characteristics of *Ceratocystis tiliae*. A. Perithecium, with an arrow indicating the collar. B. Ostiolar hyphae and emerging ascospores. C. Flask-shaped conidiophore producing cylindrical conidia. D. Cylindrical conidia and doliform conidia in a chain. E. Ascospores. F. Aleurioconidium still attached to conidiophore. All features from isolate C1954 (= CBS 137354) from the holotype, except A, which was from isolate C2622 (= CBS 137355). Bars: A = 100 μm ; B = 20 μm ; C, D, F = 10 μm ; E = 5 μm .

FOOTNOTES

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