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RESEARCH ARTICLE

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Ceratocystis quercicola sp. nov. from *Quercus variabilis* in Korea

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ABSTRACT

During a survey of putative fungal pathogens infecting oak trees in the Gangwon Province of the Republic of Korea, a fungus resembling a *Ceratocystis* sp. was repeatedly isolated from natural wounds on *Quercus variabilis*. Morphological comparisons and DNA sequence comparisons based on partial β-tubulin and TEF-1α gene regions showed that the fungus resided in a distinct lineage. This novel *Ceratocystis* species is described here as *C. quercicola* sp. nov. This is the first novel species of *Ceratocystis* to be reported from Korea. A pathogenicity test showed that it can cause lesions on inoculated trees but that it had a very low level of aggressiveness. The discovery of this fungus suggests that additional taxa residing in *Ceratocystis* are likely to be discovered in Korea in the future.

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KEYWORDS
Microascales; oak; ophiostomatoid; wound

1. Introduction

The ascomycete genus *Ceratocystis* (Microascales, Ceratocystidaceae) represents an economically important group of fungal pathogens, occurring globally on a wide range of hosts [1–3]. The genus *Ceratocystis* was introduced to accommodate *C. fimbriata*, the causal agent of black rot on sweet potato [4]. *Ceratocystis* spp. are characterized by dark, globose ascomatal bases, elongated ascomatal necks, sticky masses of ascospores accumulating at their tips, hat-shaped ascospores, and thielaviopsis-like asexual states [1].

Ceratocystis includes important fungal pathogens, some of which cause serious diseases of agricultural and forestry crops worldwide [5]. Symptoms of infection by *Ceratocystis* spp. include branch and stem cankers, vascular staining, wilt, root disease, die-back, and fruit rot [1,5]. There has been a recent and unprecedented surge of novel diseases caused by *Ceratocystis* spp. in various parts of the world. These include rapid death of *Metrosideros polymorpha* in Hawai'i caused by *C. lukuohia* and *C. huliohia* [6], canker and wilt disease of plantation-grown *Acacia* spp. in South East Asian countries caused by *C. manginecans* [7] and by *C. albifundus* in various countries of Africa [8,9].

Ceratocystis spp. require freshly made wounds as entry points to initiate infection [10–12]. The primary means of transmission to new tree hosts is by casual insects such as flies (Diptera) and nitidulid beetles (Coleoptera: Nitidulidae) visiting wounds [5,13–16]. These insects are attracted by the volatiles emanating from fresh tree wounds on which *Certocystis* spp. grow and sporulate rapidly, producing sticky spore masses that adhere to the bodies of the insects that carry them to new wounds [12,13,17,18].

A routine survey in a natural forest located in Gangneung (Gangwon province, the Republic of Korea) was conducted in 2017. This was to establish an inventory of potentially pathogenic fungi threatening the health of *Quercus* spp. One of the objectives of this study was to isolate fungi that had colonized wounded tissues resulting from severe storm damage in the summer of 2017. Among the isolated fungi was a *Ceratocystis* sp. that was consistently isolated from wounds on *Q. variabilis* (Oriental cork oak). The aim of this study was to identify this *Ceratocystis* sp. to compare it with other known *Ceratocystis* spp. and to consider its possible pathogenicity.

2. Materials and methods

2.1. Isolation

The *Ceratocystis* sp. was collected from fresh wounds on *Q. variabilis* growing in a natural forest located in Gangneung, a city in the Gangwon province of South Korea (37° 43′41.7″N 128° 47′56.1″E, 37° 43′37.4″N 128° 47′53.6″E) in July and August, 2017. Characteristic ascomata of *Ceratocystis* spp. were

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recognized using a hand lens, and pieces of bark bearing ascomata were placed in individual paper bags and transported to the laboratory for further study.

Isolations were made on 2% malt extract agar (MEA; Franklin Lakes, NJ, USA) supplemented with 100 mg/L^{-1} streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) by transferring spore drops at the apices of the ascomata onto the medium. Cultures were incubated at 25 °C for two weeks in the dark. All the isolates recovered in this study were deposited in the Culture Collection (CDH) of the National Institute of Forest Science, Seoul, South Korea, and the ex-holotype culture was deposited in the Korean Agricultural Culture Collection (KACC) of the National Academy of Agricultural Science, Jeonju, South Korea. The holotype specimen was deposited in the herbarium collection (KH) of Korea National Arboretum, Pocheon, South Korea.

2.2. Microscopy

To study the morphological characteristics, 3-week-old cultures maintained at optimum growth temperature were used. Fungal structures were mounted on microscope slides in water that was later replaced with 85% lactic acid for further observation. The structures were examined under Nikon microscopes (Eclips Ni, SMZ18; Nikon, Tokyo, Japan) mounted with Nikon camera (DS-Ri2). Fifty measurements were made for taxonomically relevant structures whenever possible. Colony color (upper and reverse surfaces) was determined using the color charts of Rayner [19].

2.3. Growth in culture

Growth of the *Ceratocystis* sp. was conducted for two isolates serving as holotype and paratype, respectively. A 5 mm mycelial plug taken from a 14-day-old culture was placed at the center of 90 mm Petri dishes containing 2% MEA. These cultures were incubated in the dark for 14 days at temperatures ranging from 5 to 35° C at 5 degree intervals. The study was repeated once with five replicate plates for each isolate at each temperature. The diameters of colonies perpendicular to each other were measured after 14 days and an averages were computed.

2.4. Genomic DNA extraction, PCR amplification, and sequencing

To extract genomic DNA, cultures were incubated for two weeks to allow sufficient mycelial growth. Mycelium was scraped from the surfaces of the agar with sterilized surgical scalpel blades and transferred to 1.5 mL Eppendorf tubes. Genomic DNA was then extracted using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research; Irvine, CA, USA) following the manufacturer's instructions. The quantity and quality of DNA extracted was evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Walthum, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). The total volume of each PCR reaction mixture was 15 µL, containing 1 µL of genomic DNA, 0.5 µL (10 pM) of each primer (forward and reverse), 0.5 µL of MyTaq PCR buffer (Bioline), and 0.5 µL of MyTaq DNA polymerase (Bioline). The PCR cycling profile consisted of an initial denaturation stage at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min.

PCR amplifications were made for two gene regions, including part of the β-tubulin 1 (BT1) using primers Bt1a and Bt1b [20], and part of the translation elongation factor-1 alpha (TEF-1α) regions with primers TEF1F and TEF2R [21]. The resulting PCR products were submitted to Macrogen (Seoul, Korea) for forward and reverse sequencing reactions.

2.5. Multi-gene phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Q. variabilis* were retrieved from GenBank. Phylogenetic trees based on a concatenated data set of the BT1 and TEF-1α gene regions were computed. Sequences for each of the two gene regions were aligned using the online interface of MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server) [22], with the iterative refinement method (FFT-NS-i settings) selected. Sequence alignments were manually edited in MEGA7 [23]. Two different phylogenetic analyses were employed, including maximum parsimony (MP) analyses using MEGA7 and maximum likelihood (ML) tests using RAxML HPC BlackBox ver.8.1.11 [24,25], using the default option with the GTR substitution model implemented in the CIPRES cluster server (https://www.phylo.org/) at the San Diego Supercomputing Center. For both MP and ML analyses, *Ceratocystis albifundus* isolate CMW 4068 (culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa) was used as the outgroup taxon.

2.6. Pathogenicity test

Pathogenicity studies were conducted on twenty freshly excised 15-cm-long × 5-cm-diameter logs of *Q. variabilis*. Bark was wounded to expose the cambium using a 10 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively

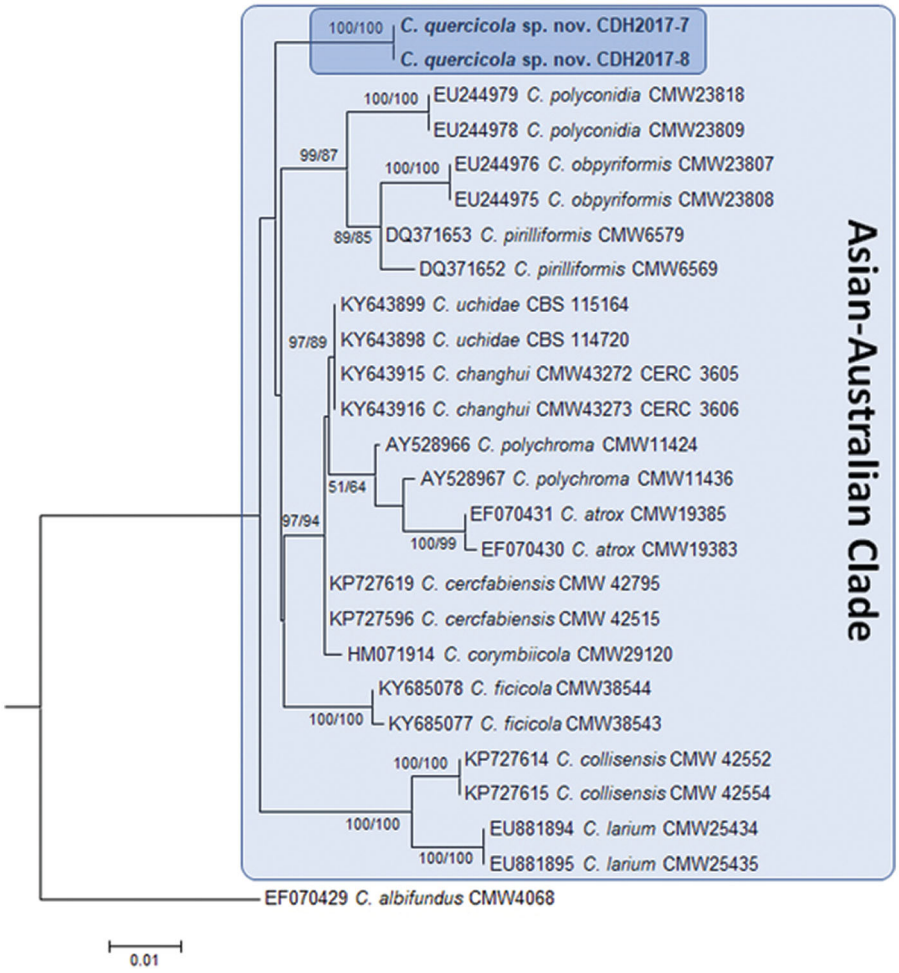

Figure 1. Phylogenetic trees based on maximum likelihood (ML) analysis of datasets of a combined dataset of BT1 and TEF-1a gene sequences for *Ceratocystis* species in the Asian-Australian clade of *Ceratocystis*. Isolates in bold and highlighted are the new species of *C. quercicola* described in this study. Bootstrap values >50% for MP and maximum likelihood (ML) are presented above branches as ML/MP, bootstrap values absent are not shown. Scale bar indicates 0.01 changes.

growing, 2-week-old cultures, (isolate CDH2017-8), were placed with the mycelium facing the cambium. Five logs were inoculated with clean agar disks to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination. The logs were placed in a moist chamber for 8 weeks. The pathogenicity test was performed twice for statistical analysis. Analysis of variance (ANOVA) and Tukey's honestly significance difference (Tukey's HSD) test were used to determine whether there were significant differences in aggressiveness of the fungus based on a P-value computed using R v.3.4.3 (RCore Team, 2017; https://www.R-project.org/).

3. Results

3.1. Multi-gene phylogenetic analyses and sequence comparisons

Two gene regions, BT1 and TEF-1α, were successfully sequenced, and these were deposited in GenBank with accession nos. MT121108–9 for BT1 and MT124072–3 for TEF-1α. The sequences obtained from BT1 and TEF-1α gene regions were aligned with closely related *Ceratocystis* spp., based on the BLAST search results from the NCBI nucleotide database BT1 (13 taxa, 545 characters), TEF-1α (13 taxa, 755 characters). Phylogenetic analyses using the concatenated BT1 and TEF-1α gene sequences resulted in a tree (Figure 1). Although the overall topologies generated from both ML and MP analyses were slightly different from each other, they consistently showed that the isolates from *Q. variabilis* represent a previously undescribed species. This species was most closely related to *C. ficicola* [26], but it was distinct from that species and all previously described *Ceratocystis* spp.

Sequence comparisons revealed that the *Ceratocystis* sp. differed from *C. ficicola* at 17 of 581 characters (about 29.3%) in the BT1 (Accession no. KY685078) and 29 of 780 characters (about 3.7%) in the TEF-1α sequences (Accession no. KY685079).

3.2. Culture characteristics and morphology

Ascomata of the *Ceratocystis* sp. from *Q. variabilis* were produced abundantly in culture. These were

characterized by dark, globose ascomatal bases with long necks and hat-shaped ascospores. Cylindrical, hyaline conidia produced in flask-shaped conidiophores, and dark aleuriospores were also common in culture (Figure 2).

3.3. Taxonomy

Morphological comparisons and phylogenetic inference based on two gene regions provided conclusively sufficient evidence to show that the *Ceratocystis* sp. isolated from *Q. variabilis* represents an undescribed species residing in the Asian-Australian clade of *Ceratocystis* [27]. This species is described as follows:

Ceratocystis quercicola D. Hyeon Lee, S.E. Cho, Marinc., M.J. Wingf., sp. nov. (Figure 2) MycoBank No. MB834642

Etymology: The epithet refers to the host genus *Quercus*, from which the fungus was collected.

Typus: Gangneung, Korea 37° 43′41.7″N 128° 47′56.1″E, isolated from freshly made wounds (less than one month) on *Quercus variabilis*. The holotype, KA20-0012, dried culture of CDH2017-8 was deposited in the herbarium collection (KH) of Korea National Arboretum. The ex-holotype culture (CDH2017-8 = KACC 48669) was deposited in the culture collection of the National Institute of Forest Science (CDH) and Korean Agricultural Culture Collection for Type Cultures (KACC).

Additional specimen examined: Mt. 293-1, Eoheul-ri, Seongsan-myeon, Gangneung-si, province. Isolated from fresh wounds on *Q. variabilis*, August 2017, D.H. Lee, culture CDH2017-7.

Habitat: Freshly wounded tissues (less than one month) of *Q. variabilis* trees.

Known distribution: The Republic of Korea (Gangwon province)

On 2% MEA, sexual and asexual states present. Sexual state. *Ascomatal base* subglobose to obpyriform, densely covered with dark hyphae, 178–269 × 141–251 µm; *ascomatal neck* straight, brown, becoming paler toward apex, 283–427 µm long, 27–41 µm wide near base, 20–29 µm wide near apex, base of neck occasionally thickened; *ostiolar hyphae* divergent, base sub-hyaline becoming hyaline toward tip, 24–58 µm long, 1–3 µm wide near base, 1–2 µm wide near apex; *ascomatal hyphae* brown, fertile or vegetative, producing 3 shapes of conidia when fertile. *Asci* evanescent. *Ascospores* hyaline, ellipsoidal to oblong with parallel sides, covered with a sheath which gives in side view a shape of hat, 5–6 × 3.5–4.5 µm (avg. 5.7 × 4.1 µm) without sheath.

Asexual state. Three different shapes of spores produced, cylindrical, barrel-shaped conidia, and aleuriospores. (1) Cylindrical; *conidiophores* macronematous, seldom branched, straight or flexuous, septate, subhyaline to hyaline, 65–195 µm long, 3–5 µm wide near base; *conidiogenous cells* endoblastic, integrated, cylindrical or tubular shape, often gradually tapering toward apex, 33–65 µm long, 3–5 µm wide near base; *conidia* abundant, hyaline, cylindrical, when produced in ascomatal hyphae, often with ends slightly bulged like a dumbbell, in chain, 8–18 × 3–4.5 µm (avg. 13 × 3.7 µm). (2) Barrel-shaped; scarce, observed only from fertile ascomatal hyphae, *conidiophores* macronematous, pigmented, straight, septate, sub-hyaline to pale brown; *conidiogenous cells* enteroblastic, integrated, cylindrical or tubular shape, becoming wider toward apex; *conidia* hyaline, in chain, barrel-shaped, 8–22 × 6–8 µm (avg. 10.3 × 7.1 µm). (3) Aleuriospores; *conidiophores* macronematous, straight or fleuxuous, occasionally branched, septate, 23–123 µm long, 3–4 µm wide near base; *conidiogenous cells* holoblastic, integrated, hyaline or subhyline, cylindrical or tubular, 11–23 µm long, 3.5–4 µm wide; *conidia* abundant, terminal, in chain, basauxic, ellipsoidal to sub-globose, occasionally with base elongated, hyaline when young, becoming pigmented with age, 11–16 × 7.5–12 µm (avg. 13.5 × 9 µm).

Culture characteristics: Colonies on MEA greenish olivaceous, reverse greenish olivaceous. Mycelium immersed and superficial. Hyphae smooth, septate, without constriction at septa. Colony surfaces scattered with black ascomata. Optimal temperature for growth 25 ° C reaching 75 mm in 14 days, followed by 30 ° C (53 mm) and 20 °C (51 mm). No growth at 5 or 35 ˚C.

Notes: *Ceratocystis quercicola* was phylogenetically closely related to *C. ficicola* which first reported in Japan causing canker on *Ficus carica* (fig) [26]. However, *C. ficicola* had much larger structures than *C. quercicola*; larger ascomata (*C. ficicola*: 280–640 µm wide, *C. quercicola*: 141–251 µm wide), longer ascomatal neck (*C. ficicola*: 890–2,460 µm long, *C. quercicola*: 283–427 µm long), longer ostiolar hyphae (*C. ficicola*: 140–300 µm, *C. quercicola*: 24–58 µm long), and larger ascospores (*C. ficicola*: 6.5–8 × 4–5.5 µm, *C. quercicola*: 5–6 × 3.5–4.5 µm).

3.4. Pathogenicity test

The isolate used in the pathogenicity study produced distinct lesions on the cambium of the *Q. variabilis* logs 8 weeks after the inoculation (Figure 3). This is in contrast to the control inoculations where there were no signs of infection (p value < 0.01, at the 95% confidence level). Re-isolations from the lesions resulted in *C. quercicola* isolates morphologically indistinguishable from those inoculated onto the logs.

Figure 2. Field and microscopic features of *Ceratocystis quercicola* sp. nov. (ex-holotype: KACC 48699). (A) The wounded oak tree, *Quercus variabilies* from which the fungus was isolated. (B) Close-up of wound showing dark stain and fungal growth. (C) Fruiting structures on 2% MEA showing asexual states (white masses and thread-like) and a cluster of ascomata characterized by masses of dark hyphae (arrow). (D) Ascoma with a neck with thickened base. (E) Ascoma densely covered with dark fertile hyphae. (F,G) Divergent Ostiolar hyphae. (H,I) Ascospores with evident of gelatinous sheath (arrow). (J,K) Conidiogenous cell producing barrel-shaped conidia. (L) Chain of barrel-shaped conidia. (M) Aleuriospores showing basauxic generation. (N) Some alurioconidia on 2% MEA with elongated base. (O) Chain of Aleuriospores. (P) Conidiophores producing cylindrical shape conidia. (Q,R) Conidiogenous cell producing cylindrical shape conidium. (S,T) Cylindrical shape conidia. Scale bars: *C* = 500 μm; D, *E* = 100 μm; F, G, L–P, S, *T* = 10 μm; H–K, Q, *R* = 5 μm.

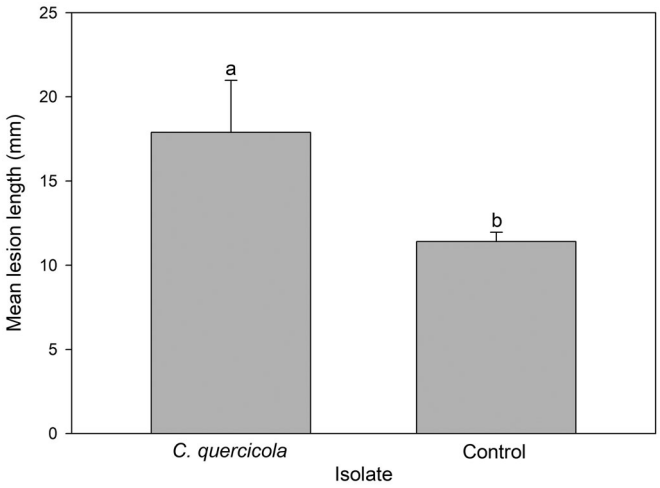

Figure 3. Mean lesion lengths on freshly cut bolts of *Quercus variabilis* 8 weeks after inoculation with *Ceratocystis quercicola* (isolate CDH2017-8). Sterile agar discs were used in control inoculations. Bars represent standard deviation of the mean, and it was annotated with the different letters to indicate that it is significantly different at $p < 0.01$.

4. Discussion

A routine tree health survey in a natural forest of Korea resulted in the discovery of a new *Ceratocystis* sp., commonly occurring on fresh wounds of *Q. variabilis*. Phylogenetic inference based on sequence data for parts of the BT1 and TEF-1α gene regions showed that this species resides in the Asian-Australian Clade of *Ceratocystis* [27]. The new species was provided with the name *Ceratocystis quercicola*, which was shown to be most closely related to *C. ficicola*. Other than being distinct from *C. ficicola* based on DNA sequences, *C. quercicola* can easily be distinguished from other *Ceratocystis* spp. based on its morphological characteristics.

The presence of *C. quercicola* on freshly induced wounds on trees is typical for *Ceratocystis* spp. as well as various other genera in the Ceratocystidaceae as defined by de Beer et al. [1]. We assume that, as with other *Ceratocystis* spp. and their relatives [5,13–16], *C. quercicola* was transferred to the wounds on *Q. variabilis* by insects such as flies and nitidulid beetles. This hypothesis needs to be tested.

The relatedness of *C. quercicola* and *C. ficicola* was not surprising given the fact that both fungi occur on trees in southeast Asia. However, *C. ficicola* is an aggressive pathogen of *Ficus carica* in Japan [26] and the disease that it causes in that country suggests that it could be an introduced pathogen. The pathogenicity test conducted in this study showed evidence that *C. quercicola* might be a pathogen, given the fact that lesions on inoculated trees of *Q. variabilis* are developed by infection of the fungus with very low level of aggressiveness. However, the preliminary pathogenicity test was conducted on freshly excised *Q. variabilis* bolts and the results should be interpreted with caution. Ideally, living trees would be inoculated but these could not be sourced and a proxy for pathogenicity [28].

Very little is known regarding species of Ceratocystidaceae or other insect related fungi such as the Ophiostomatales in Korea. The results of this study illustrate the fact that there is a significant reason to establish a baseline of understanding regarding these fungi in the country. Further surveys and research is planned to achieve this goal in the future.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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