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Ceratocystis quercina sp. nov. (Ceratocystidaceae) from *Quercus mongolica* in South Korea

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Abstract

During a survey of putative tree pathogens on oak forests in Gangwon Province, South Korea, a fungal isolate exhibiting morphological characteristics typical of *Ceratocystis* was consistently recovered from natural wounds on *Quercus mongolica*. Multi-locus phylogenetic analyses based on maximum parsimony and maximum likelihood approaches using three loci (*TUB2*, *TEF1α* and *MCM7*), combined with distinctive morphological features of aleuriospores and conidia, revealed that the isolate forms a phylogenetically distinct lineage. Consequently, this fungus is herein described as *Ceratocystis quercina sp. nov.*, marking the second novel species newly described from South Korea, following *C. quercicola*. In this regard, this discovery suggests the potential for further taxonomic novelty within the genus *Ceratocystis* and highlights the need for broader surveys to determine the actual prevalence and spatial distribution of *Ceratocystis* taxa in the Korean Peninsula.

Key words: Ophiostomatoid, Microascales, oak, insect vector, morphology, taxonomy, phylogeny

Introduction

The genus *Ceratocystis* (Microascales, Ceratocystidaceae) consists of fungi ranging from weakly pathogenic wound colonizers of trees to high destructive fungal pathogenic groups affecting woody and herbaceous plant hosts worldwide. They are leading to serious diseases such as wilt, canker, and rot in plantation forestry and agricultural crops (Wingfield *et al*. 2013, de Beer *et al*. 2014). Representative examples include *C. fimbriata*, which is well known as the causal agent of post-harvest black rot of sweet potato (*Ipomoea batatas*) (Baker *et al*. 2013, Li *et al*. 2016). In addition, *C. manginecans* and *C. platani* are the causative agent responsible for severe canker and wilt diseases in *Acacia mangium* (Tarigan *et al*. 2011, Brawner *et al*. 2015) and *Platanus orientalis* (Engelbrecht *et al*. 2004, Tsopelas *et al*. 2017), respectively.

Typified by *C. fimbriata*, the causal agent of black rot on sweet potato (Halsted 1890), members of this genus are morphologically distinguished by dark, globose ascomata with elongated necks that exude hydrophobic masses of hat-shaped ascospores at their tips (Wingfield *et al*. 2013). *Ceratocystis* species are wound-colonizing pathogens that require fresh injuries to initiate infection (DeVay *et al*. 1968, Teviotdale *et al*. 1991). Transmission is facilitated by non-specific insect associates, including dipterans and nitidulid beetles, which are drawn to host volatiles emanating from damaged tissues (Moller *et al*. 1968, Juzwik *et al*. 2004, Heath *et al*. 2009). Upon visiting these sites, the insects acquire adhesive spore masses produced by the fungus and subsequently vector the inoculum to healthy trees (Juzwik & French 1983, Kile 1993, Wingfield *et al.* 2017a, Holland *et al*. 2019).

Despite the economically and ecologically importance of the genus, only one species, *C. quercicola* (Cho *et al.* 2020), has to date been formally reported from South Korea, and the diversity and distribution of *Ceratocystis* species on broadleaved trees in the country remain largely unexplored. To begin closing this gap, and as part of an

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effort to establish an inventory of ophiostomatoid fungi potentially threatening the health of *Quercus* spp. in South Korea, a survey was undertaken in the natural forests of Gangneung, Gangwon Province, South Korea, in 2023. This was achieved by investigating naturally wounded tissues colonized by fungi, especially resulting from storm damage in the summer season. During the survey, a *Ceratocystis* species was consistently isolated from wounded tissues of *Quercus mongolica*. Consequently, the aim of this study was to clarify the taxonomic identity of this isolate through comparisons with known other *Ceratocystis* species based on morphology and phylogenetic inferences.

Materials and methods

Isolation

The *Ceratocystis* sp. was collected from fresh wounds on *Q. mongolica*, the most common tree species, growing in the natural forests located in Gangneung, a city in the Gangwon Province of South Korea (37°43'34.2"N 128°47'58.0"E) during May and September 2023. Pieces of bark bearing fungal mats were placed in individual paper bags and transported to the laboratory for further study.

Fungal isolations were achieved by transferring spore drops at the apices of the ascomata to the medium on 2% malt extract agar (MEA; 20 g malt extract, Difco; 20 g agar, Difco) supplemented with 100 mg L^{-1} streptomycin sulphate (Sigma-Aldrich). Cultures were then incubated at 25 °C for two weeks in the dark. All the isolates successfully recovered in this study were deposited in the Culture Collection (CDH) of the Chungnam National University, Daejeon, South Korea (Accession Nos. CDH137 and CDH137-2). The holotype specimen, CDH137, was deposited in the herbarium collection (KH) of Korea National Arboretum, Pocheon, South Korea (Accession No. KA25-1571), and the ex-holotype culture was deposited in the Korean Agricultural Culture Collection (KACC) of the National Academy of Agricultural Science, Jeonju, South Korea (Accession No. KACC 411102).

Microscopy

For morphological inspections, fungal structures from the 3-week-old cultures grown on 2% MEA at the optimal growth temperature were mounted in water on microscope slides, that was later replaced with 85% lactic acid for further observation. Microscopic examination and digital imaging were performed using Zeiss AX10 Imager A2 (Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with an Axiocam 506 digital camera. Biometric measurements (n = 30) were taken for taxonomically relevant structures wherever possible.

Genomic DNA extraction, PCR amplification, and sequencing

Mycelium was harvested from 2-week-old cultures by scraping the agar surface with a sterilized scalpel. The harvested mycelium was transferred to 1.5 mL microcentrifuge tubes for subsequent genomic DNA extraction. This was achieved using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The quantity and quality of the DNA extracted was evaluated with a microvolume spectrophotometer (OPTIZEN NanoQ; KLAB, Daejeon, South Korea) to calibrate the concentration and purity of DNA as PCR templates.

PCR amplification was performed using a WN400 thermal cycler (Clontech, CA, USA) in a total reaction volume of 20 µl. The reaction mixture contained 2 µl of genomic DNA, 0.5 µl (10 pM) of each primer, 10 µl of Platinum™ Direct PCR Universal Master Mix (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and 7 µL of nuclease-free water (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 8 min.

For phylogenetic inferences of *Ceratocystis* species, partial regions of six loci, were amplified and sequenced. These were achieved based on the Internal Transcribed Spacer (ITS) rDNA regions and the 5.8S rRNA gene using the primers ITS1F/ITS4 (White *et al*. 1990, Gardes & Bruns 1993), Beta-tubulin 2 (*TUB2*) gene using primers Bt1a/Bt1b (Glass & Donaldson 1995), Transcription Elongation Factor-1 alpha (*TEF1α*) gene with primers TEF1F/TEF2R (Jacobs *et al*. 2004), actin (*ACT*) using ACT-513F/ ACT-783R (Carbone & Kohn 1999), the mini-chromosome maintenance complex component 7 (*MCM7*) using Cer-MCM7F/Cer-MCM7R (de Beer *et al*. 2014) and second largest subunits of

RNA polymerase II (*RPB2*) using degenerate primers RPB2-5Fb/RPB2-7Rb (Fourie *et al*. 2015). The resulting PCR products were then submitted to Macrogen (Seoul, South Korea) for forward and reverse sequencing reactions.

Multi-gene phylogenetic analyses

The ten sequences of *Ceratocystis* spp., most closely related to the isolates obtained from *Q. mongolica* in this study, were retrieved using BLASTn searches against from GenBank (Table 1). The concatenated sequences were aligned with MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh *et al*. 2019) with G-INS-1 method and 1PAM/κ = 2 scoring matrix (Katoh *et al*. 2005). Alignments were edited and trimmed in MEGA version 7 (Kumar *et al*. 2016), and were then used as input for phylogenetic tree construction.

TABLE 1. *Ceratocystis* species used for phylogenetic analyses in this study.

Species	Isolate	Gene regions		
		TUB2	*TEF1α*	*MCM7*
C. betulina	C1709/CBS 144246[T]	MG980839	MG980743	MG980985
	C1770	MG980840	MG980744	MG980986
C. caryae	C1829/CBS 114716[T]	MG980829	MG980733	MG980978
	C1827/CBS 115168	MG980828	MG980732	KM495414
C. destructans	KARE1428/CBS144247[T]	MG980860	MG980764	MG981006
	C578	MG980845	MG980749	MG980991
C. fimbriata	C1476/C1099	MG980827	MG980731	MG980977
C. harringtonii	C685/CBS 115161[T]	MG980832	MG980736	MG980980
	C1485/ATCC 24096	MG980833	MG980737	MG980981
C. smalleyi	C684/CBS 114724[T]	MG980830	MG980734	KM495463
	C682	MG980831	MG980735	MG980979
C. tiliae	C2131/CBS 137355[T]	MG980875	MG980779	MG981021
	C1954/CBS 137354	MG980874	MG980778	MG981020
C. variospora	C1009/CBS 773.73[T]	MG980835	MG980739	MG980982
	C1843/CBS 114715	MG980837	MG980741	KM495471
C. quercicola	CDH2017-7[T]	MT121108	MT124072	-
	CDH2017-8	MT121109	MT124073	-
C. quericina	**CDH137[T]**	**PX964240**	**PX964238**	**PX964234**
	CDH137-2	**PX964241**	**PX964239**	**PX964235**
Thielaviopsis cerberus	CMW 36668[T]	JX518380	JX518316	KM495415

[1]Ioslates in bold represent isolates obtained in this study.

[2]T = Type specimens used in the phylogenetic analyses.

For phylogenetic analyses, both maximum parsimony (MP) and maximum likelihood (ML) approaches were employed. Most parsimonious trees were obtained using a heuristic search and TBR branch swopping strategy based on 1000 repeats. ML phylogenetic trees were constructed with the TN93 +R model using the default option via PhyML 3.0 (https://website.atgc-montpellier.fr/tool/phyml-3-0/) (Guindon & Gascuel 2003; Guindon *et al*. 2010) with 1,000 bootstrap replicates to assess branch support. For both MP and ML analyses, *Thielaviopsis cerberus* isolate CMW 36668, was used as the outgroup taxon.

Results

Sequencing and multi-gene phylogenetic analyses

Six gene regions, (ITS, *TUB2*, *TEF1α*, *ACT*, *MCM7*, and *RPB2*), were successfully sequenced, and the resulting sequences were deposited in GenBank with accession numbers. PX972485–486 for ITS, PX964240–241 for *TUB2*, PX964238–239 for *TEF1*, PX964236–237 for *ACT*, PX964234–245 for *MCM7*, and PX964232–233 for *RPB2*.

To determine the phylogenetic position of the isolate, we initially performed the BLAST searches and subsequently constructed a phylogenetic tree based on the ITS gene region. Based on these results, we selected closely related taxa, ensuring the inclusion of all available representative *Ceratocystis* species that possess sufficient sequence data for the *TUB2*, *TEF1α*, and *MCM7* gene regions (Table 1). The sequences obtained were then aligned with those of representative *Ceratocystis* species, and phylogenetic analyses were performed based on both MP and ML.

Although the overall topologies generated from both ML and MP analyses were slightly different from each other, it was clearly represented that the isolates from *Q. mongolica* formed a monophyletic lineage with high bootstrap support values (Figure 1). Although this species was phylogenetically most closely related to *C. destructans* (Holland *et al*. 2019), it was distinct from all other described *Ceratocystis* species.

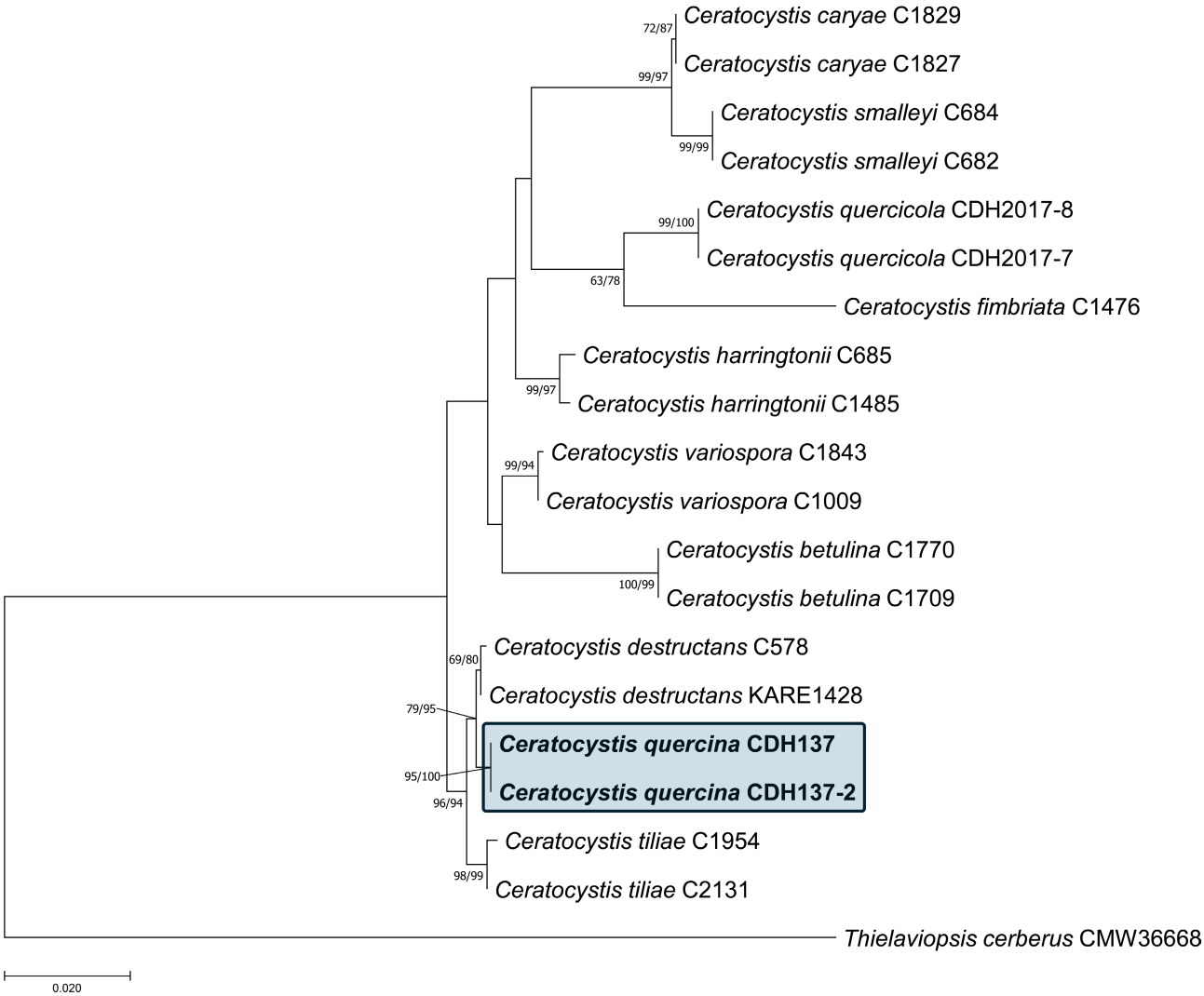

FIGURE 1. Phylogenetic trees based on maximum likelihood (ML) analysis of datasets of a combined dataset of *TUB2*, *TEF1α*, and *MCM7* gene sequences for *Ceratocystis* species and its close related species. Isolates in bold are the new species of *C. quercina* described in this study. Bootstrap values >50% for MP and ML are presented above branches as MP/ML. Scale bar indicates 0.02 changes.

In addition, sequence comparisons revealed that the novel species differed from *C. destructans* at 7 of 545 characters excluding gaps (about 1.3%) in the *TUB2* (Accession no. MG980841), at 12 of 766 characters excluding gaps (about 1.6%) in the *TEF1α* (Accession no. MG980745), and at 3 of 612 characters excluding gaps (about 0.5%) in the MCM7 sequences (Accession no. MG980987).

Taxonomy

Morphological comparisons and the phylogenetic tree constructed based on MP and ML using the combined three-gene dataset provided sufficient evidence that the isolates from fresh wounds on *Q. mongolica* represent an undescribed species that belongs to the genus *Ceratocystis*. This species is described as follows:

Ceratocystis quercina H.G. Woo, D.Hyeon Lee & S.E. Cho, *sp. nov.* **Figure 2**
Mycobank: MB 862350.

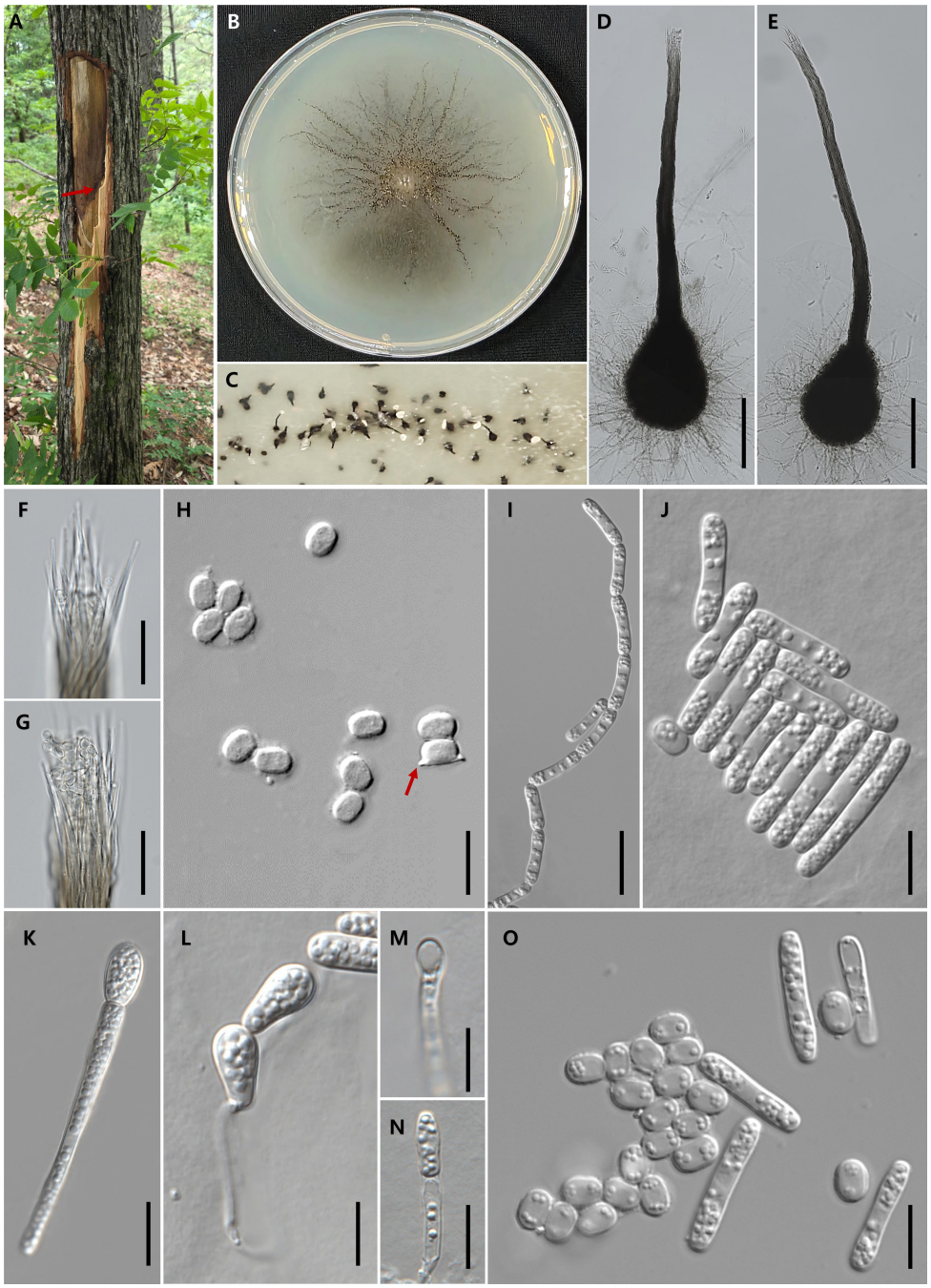

FIGURE 2. Field, cultural and microscopic features of *Ceratocystis quercina sp. nov.* (ex-holotype: KACC 411102). (A) The wounded oak tree, *Quercus mongolica* from which the fungus was isolated (arrow). (B) Fungal colony on 2% MEA. (C) Fruiting structures on 2% MEA showing a cluster of ascomata. (D,E) Ascoma with a neck with thickened base. (F) Divergent ostiolar hyphae. (G) Ostiolar hyphae producing ascospores. (H) Ascospores with evident of gelatinous sheath giving hat-shaped appearance in side view (arrow). (I) Chain of cylindrical shape conidia. (J) Cylindrical shape conidia. (K) Aleuriospore showing basauxic generation. (L) Chain of aleuriospores. (M) Conidiogenous cell producing barrel-shaped conidia. (N) Conidiogenous cell producing cylindrical shape conidia (O) Barrel-shaped conidia and cylindrical shape conidia. Scale bars: D, E = 100 µm; F, G = 20 µm; H, J, M, N, O = 10 µm; I, L = 20 µm; K = 5 µm.

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

Type: Republic of Korea: Gangwon, Gangneung, Seongsan-myeon, Eoheul-ri, Mt. 293-1; 37°43'34.2"N 128°47'58.0"E. Wounds, *Quercus mongolica*. 1 Sep. 2023. *D.Hyeon Lee.* CDH137 (**Holotype** KA25-1571, ex-type living culture KACC 411102).

Description: On 2% MEA, sexual and asexual states present. Sexual morph. *Ascomata* developing at 25 °C for two weeks in the dark; perithecial, with a long ostiole, solitary to aggregated, semi-immersed to superficial on the culture media *Ascomatal base* subglobose to obpyriform, densely covered with dark hyphae, 147–194 × 117–146 µm (n = 30); *Ascomatal neck* straight, brown, becoming paler toward apex, 473–510 µm long, 21–33 µm wide near base, 15–22 µm wide near apex, base of neck occasionally thickened (n = 30); *Ostiolar hyphae* divergent, aseptate, base sub-hyaline becoming hyaline towards the tip, 37–71 µm long, 1–3 µm wide near base, 1–2 µm wide near apex (n = 30); *Asci* evanescent. *Ascospores* hyaline, ellipsoidal to oblong with parallel sides, aseptate, covered with a sheath which gives in side view a shape of hat, 4.8–5.7 × 3.2–4.1 µm (avg. 5.1 × 3.6 µm ; n = 30) without sheath.

Asexual morph. Three different shapes of spores produced, cylindrical, barrel-shaped conidia, and aleuriospores. (1) Cylindrical; *conidiophores* macronematous, seldom branched, straight or flexuous, septate, hyaline, 69–207 µm long, 2.5–5 µm wide near base; *conidiogenous cells* endoblastic, integrated, cylindrical or tubular shape, often gradually tapering toward apex, 39–75 µm long, 2.5–5 µm wide near base; *conidia* abundant, hyaline, cylindrical, either singly or in chain, 12.7–24.6 × 2.6–4.7 µm (avg. 18.2 × 3.4 µm; n = 30). (2) Barrel-shaped; *conidiophores* macronematous, pigmented, straight, septate, sub-hyaline to pale brown; *conidiogenous cells* enteroblastic, integrated, cylindrical or tubular shape, becoming wider toward apex; *conidia* abundant, hyaline, barrel-shaped, 4.7–5.8 × 3.3–4.3 µm (avg. 5.3 × 3.7 µm; n = 30). (3) Aleuriospores; *conidiophores* macronematous, straight or fleuxuous, occasionally branched, 31–132 µm long, 2.3–4.3 µm wide near base; *conidiogenous cells* holoblastic, integrated, hyaline or subhyline, cylindrical or tubular, 14.5–27.8 µm long, 2.5–4.1 µm wide; *conidia* abundant, terminal, in chain, basauxic, ellipsoidal to sub-globose, occasionally with base elongated, hyaline, not pigmented, 9.3–10.1 × 5.1–5.4 µm (avg. 9.3 × 5.2 µm; n = 30).

Culture characteristics. *Colonies* 35 mm after 7 d at 25 °C on MEA, grayish olivaceous, reverse grayish olivaceous, turning dark olive brown with age, *Mycelium* sparse, immersed and superficial. Hyphae smooth, septate, without constriction at septa

Additional specimen examined: Republic of Korea: Gangwon, Gangneung, Seongsan-myeon, Eoheul-ri, Mt. 293-1; 37°43'34.2"N 128°47'58.0"E. Wounds, *Quercus mongolica*. 19 Sep. 2023. *D.Hyeon Lee.* (**Paratype** CDH137-2).

Habitat: Fresh wounds (less than one month-old) on *Quercus mongolica* trees.

Notes: *Ceratocystis quercina* (CDH137 and CDH137-2) is phylogenetically closely related to *C. destructans* (KARE1428), but differs in several morphological characteristics. Most notably, *C. quercina* possesses hyaline and narrower aleuriospores compared to the dark brown and wider aleurioconidia *C. destructans*. Additionally, the cylindrical conidia of *C. quercina* are generally wider, while its barrel-shaped conidia are smaller than those of *C. destructans* (Holland *et al*. 2019).

Discussion

As part of a survey aimed at establishing an inventory of the ophiostomatoid fungi that potentially pose a threat to the health of broadleaved trees in a natural forest of South Korea revealed a novel *Ceratocystis* species, isolated from fresh wounds on *Quercus mongolica*. The novel species was formally described as *Ceratocystis quercina*, exhibiting the closest phylogenetic affinity to *C. destructans* within the North American clade of *Ceratocystis* (Holland *et al*. 2019). Despite this close relationship, it is clearly separable from *C. destructans* based on multi-locus DNA sequence data and can be reliably distinguished from other members of the genus by distinct morphological characteristics, including hyaline aleuriospores and smaller barrel-shaped conidia.

The occurrence of *C. quercina* on freshly formed wounds is typical of *Ceratocystis* species, as well as several other genera within the Ceratocystidaceae as defined by de Beer *et al*. (2012). Similar to other *Ceratocystis* species and their close relatives (Moller *et al*. 1968, Juzwik *et al*. 2004, Heath *et al*. 2009; Wingfield *et al.* 2017a), *C. quercina* is presumed to be transmitted to wounds on *Q. mongolica* by insect vectors, including flies and nitidulid beetles. However, this transmission pathway requires further experimental verification.

The close phylogenetic relationship between *C. quercina* and *C. destructans*, both of which belong to the North American clade, was unexpected. Despite their shared placement within the same phylogeographic lineage, the two species are currently associated with woody hosts in geographically distant regions, including South Korea and the USA. Although multi-gene sequence analyses may offer the higher resolution required to delimit species more clearly and to infer such dispersal events, their use remains constrained in certain *Ceratocystis* clades. In particular, while partial sequences for mating-type loci are available (Holland *et al.* 2019), universal barcoding genes for *Ceratocystis* species, including *ACT*, *RPB2* and *MAT* loci, remain notably absent in these lineages (Cho *et al.* 2020).

Notably, *C. quercicola* (Cho *et al*. 2020) and *C. quercina* were isolated from the same geographic region in South Korea; however, they were assigned to different phylogeographic lineages. While the recovery of only two *C. quercina* isolates in the current study limits our understanding of its overall abundance and spatial distribution, the co-occurrence of these distinct evolutionary lineages in a single region highlights the potential for complex, and both intercontinental introduction and intracontinental expansion among *Ceratocystis* species (Ploetz *et al.* 2013; Wingfield *et al.* 2017b). Accordingly, broader and more intensive surveys incorporating extensive regional sampling and multi-locus phylogenetic analyses are required to determine the actual prevalence of these species, more accurately resolve species boundaries, and clarify evolutionary relationships and biogeographic patterns within *Ceratocystis*.

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