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Morphological and Phylogenetic Analyses Reveal a New Species of *Ceratocystiopsis* (*Ophiostomataceae, Ophiostomatales*) Associated with *Ips subelongatus* in Inner Mongolia (China) with Weak Host Pathogenicity

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Abstract: Ophiostomatoid fungi are known for their associations with bark beetles, and some species are important sources of tree diseases. *Ceratocystiopsis* is a genus of the ophiostomatoid fungi in order *Ophiostomatales*. The shortage of DNA barcodes for many species in this genus has resulted in the presence of many unnamed cryptic species. In this study, *Ceratocystiopsis subelongati* sp. nov. associated with *Ips subelongatus* infesting *Pinus sylvestris* var. *mongolica* in Inner Mongolia, China, was identified and described based on phylogenetic inference of multi-gene DNA sequences and morphological characteristics. The species is characterized by a hyalorhinocladiella- to sporothrix-like asexual state and an optimal growth temperature of 30 °C. Artificial inoculation tests in the field showed that it is mildly pathogenic to five-year-old larch trees, the main host of *I. subelongatus*. It is also the first described *Ceratocystiopsis* species associated with *I. subelongatus* in China. This discovery should provide new avenues for studying the symbiosis between bark beetles and ophiostomatoid fungi.

Keywords: bark beetle; *Ceratocystiopsis subelongati*; conifer; ophiostomatoid fungi; *Pinus*

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1. Introduction

Ophiostomatoid fungi belong to the *Ascomycota* (orders *Ophiostomatales* and *Microascales*), which have similar basic morphological features, such as ascomata with long necks and sticky drops on the conidiogenous apparatus [1,2]. These morphological features are thought to represent convergent evolution to be better transmitted by vector insects [3]. Many of these species can form symbiotic relationships with bark and ambrosia beetles [4,5], mainly because the fungi can provide nutrients for and emit the pheromones of bark beetles [6–13].

Ceratocystiopsis is a member of the family *Ophiostomataceae* and was originally described by Upadhyay and Kendrick [14]. The genus was thought to be synonymous with *Ophiostoma* for some time [15], until Zipfel et al. [16] reinstated it and distinguished it from other genera in the *Ophiostomatales* by the presence of short-necked ascomata and elongated, falcate, sheathed ascospores [1]. A total of 13 species and five unnamed cryptic species have been confirmed based on phylogenetic analyses of the nuclear ribosomal large subunit region (LSU) sequences [1]. Subsequently, *C. synnemata*, *C. lunata*, *C. yantaiensis*, and *C. weihaiensis* were identified and described based on internal transcribed spacer regions 1 and 2 of the nuclear ribosomal DNA operon, including the 5.8S region (ITS), the β-tubulin gene region (Tub2), and the transcription elongation factor1-α gene region

(TEF1-α) sequences by Strzałka et al. [17], Nel et al. [18], and Chang et al. [19]. However, there remains a shortage of DNA barcodes for many species in this genus, resulting in the persistence of many unnamed cryptic species [1,20] and posing a challenge for developing a unified taxonomy of the genus.

Although the idea that the pathogenic fungi associated with bark beetles are critically important for overcoming host tree defenses has been challenged in the literature [21], none deny that some of these fungi are important phytopathogens of forest diseases [22–25]. The best-known examples are Dutch elm disease [24,26], black stain root disease [27], and laurel wilt [28], which are caused by ophiostomatoid fungi associated with ambrosia or bark beetles. In China, a total of 44 new ophiostomatoid fungi species associated with several bark beetles infesting conifers have been reported in the last three years [19,20,29–37]. However, their pathogenicity in their respective hosts remains unknown. In this study, we aimed to elucidate the identity of the unnamed taxa *Ceratocystiopsis* cf. *pallidobrunnea* associated with *Ips subelongatus* collected during previous surveys from Northeastern China [20], based on morphological and multilocus phylogenetic methods. Additionally, we tested the pathogenicity of this species in larch, the main host of the beetle in the field.

2. Materials and Methods

2.1. Fungal Strains

Strains were isolated from galleries of *I. subelongatus* infesting *Pinus sylvestris* var. *mongolica* in Inner Mongolia, China. Other sampling and isolate details followed the descriptions of Wang et al. [20]. The strains were deposited at the China Forestry Culture Collection Center (CFCC) in Beijing and Shandong Agricultural University in Tai'an, Shandong province.

2.2. Morphological and Cultural Studies

The microscopic features of the studied fungal strains were observed and recorded by using an Olympus SZX16 stereomicroscope and an Olympus DP70 digital camera (Olympus Corp., Beijing, China). The lengths and widths of 30 reproductive structures per strain were measured in 80% lactic acid on glass slides and presented as minima, averages (±standard deviations), and maxima. A mycelium disk (5 mm diameter) cut from an actively growing culture was placed in the center of a 90 mm–diameter Petri dish containing 2% malt extract agar (MEA, AoBoXing Company Ltd., Beijing, China), which was used to measure growth rates. Five replicate plates were incubated in the dark at 5–40 °C with different treatments at 5 °C intervals. Two colony diameters perpendicular to each other were measured and recorded daily until the mycelium reached the margin of the MEA plates. The color descriptions were based on Rayner's [38] charts.

2.3. DNA Extraction, PCR, and Sequencing

Fungal strains were grown in 2% MEA at 25 °C for 10 days before DNA extraction. The actively growing mycelium was scraped from the surface of the MEA and transferred into 2 μL Eppendorf tubes. DNA extraction was performed by using an Invisorb Spin Plant Mini Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The primers ITS1 and ITS4 [39] were used to amplify ITS, Bt2a and Bt2b [40] were used to amplify Tub2, and EF1F and EF2R were used to amplify TEF1-α [41].

Polymerase chain reaction (PCR) was conducted by using the 2 × Tap PCR MasterMix (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The PCR conditions for the two regions were as follows: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 45 s at 55 °C (ITS) or 56 °C (Tub2 and TEF1-α), and 1 min at 72 °C, and a final elongation step at 72 °C for 8 min. The PCR products were transported to the Majorbio Company (Beijing, China) for sequencing.

2.4. Phylogenetic Analysis

Referenced sequences of *Ceratocystiopsis* spp. in the analyses were downloaded from GenBank (Table 1). Alignments were performed by using the online tool MAFFT v. 7 [42] with iterative refinement methods (L-INS-i). Molecular evolutionary genetic analyses (MEGA) v. 7.0 [43] was used to compile our datasets, while maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods were used for phylogenetic analyses. For the combined gene dataset, PAUP v. 4.0b10 [44] was performed to homogeneity test before phylogenetic analysis. Maximum likelihood analyses were performed by using RAxML-HPC v.8.2.3 [45] with the GTR + G model of site substitution, including estimates of gamma-distributed rate heterogeneity and proportions of invariant sites [46]. A total of 1000 trees were retained, and bootstrap support values were estimated with 1000 replicates.

Table 1. Information of strains used for phylogenetic analysis in this study.

Species Name [1]	Strain Number [2,3]	Host [4]	Vector [5]	Origin	GenBank Accession No. [6]	
					ITS	Tub2
Ceratocystiopsis brevicomi	CBS333.97	Unknown	*Dendroctonus brevicomis*	USA	EU913722	EU913761
C. collifera	CBS126.89	*Pinus teocote*	*D. valens*	Mexico	EU913721	EU913760
C. longispora	UM48	*P. banksiana*	Unknown	Canada	EU913723	-
C. lunata	CMW55897	Unknown	*Xylosandrus crassiusculus*	South Africa	MW028169	MW066754
	CMW55898	Unknown	*X. crassiusculus*	South Africa	MW028170	MW066755
C. manitobensis	UM214	*P. resinosa*	Unknown	Canada	EU913715	EU913754
	UM237	*P. resinosa*	Bark beetle	Canada	EU913714	EU913753
C. minima	UM85	*P. resinosa*	Bark beetle	Canada	EU913701	EU913740
C. minuta	CBS116796	*Picea abies*	*Ips typographus*	Poland	EU913695	EU913734
	UM1532	*Pi. abies*	*I. typographus*	Poland	EU913697	EU913736
C. minuta-bicolor	UM480	*P. contorta*	Bark beetle	Canada	EU913705	EU913744
	CBS635.66	*P. contorta*	*Ips* sp.	USA	EU913706	EU913745
C. pallidobrunnea	UM51	*Populus tremuloides*	Unknown	Canada	MN901004	MN901013
C. ranaculosa	CBS216.88	*P. teocote*	*D. frontalis*	USA	EU913713	EU913752
C. rollhanseniana	UM110	*P. sylvestris*	Beetle	Norway	EU913719	EU913758
	UM113	*P. sylvestris*	Beetle	Norway	EU913718	EU913757
C. subelongati	**CFCC52689 T**	***P. sylvestris* var. *mongolica***	***I. subelongatus***	**China**	**OL605962**	**OL622040**
	CFCC52690	***P. sylvestris* var. *mongolica***	***I. subelongatus***	**China**	**OL605963**	**OL622041**
C. synnemata	KFL16918DA	*Po. tremula*	*Dr. alni*	Poland	MN900988	MN901009
	KFL17718DA	*Po. tremula*	*Dr. alni*	Poland	MN900989	MN901010
Ceratocystiopsis sp. 1	WY13TX1-3	*P. contorta*	*D. ponderosae*	Canada	EU913707	EU913746
	WY21TX1-2	*P. contorta*	*D. ponderosae*	Canada	EU913708	EU913747
Ceratocystiopsis sp. 2	YCC329	*Larix kaempferi*	*I. subelongatus*	Japan	EU913711	EU913750
	YCC330	*L. kaempferi*	*I. subelongatus*	Japan	EU913710	EU913749
Ceratocystiopsis sp. 3	SWT1	*Pi. glauca*	*I. perturbatus*	Canada	EU913716	-
	SWT3	*Pi. glauca*	*I. perturbatus*	Canada	EU913717	-
C. weihaiensis	SNM634	*P. thunbergii*	*Cryphalus piceae*	China	MW989412	MZ019524
	SNM639	*P. thunbergii*	*Cr. piceae*	China	MW989413	MZ019525
C. yantaiensis	SNM582	*P. thunbergii*	*Cr. piceae*	China	MW989410	MZ019522
	SNM650	*P. thunbergii*	*Cr. piceae*	China	MW989411	MZ019523
Ophiostoma ips	CBS137.36	Unknown	*Ips* sp.	USA	EU913685	EU913724

[1] Species names in bold are novel species described in this study. [2] CFCC: China Forestry Culture Collection Center, Beijing, China. [3] T = ex-holotype isolate. [4] *P.*, *Pinus*; *Pi.*, *Picea*; *Po.*, *Populus*; *L.*, *Larix*. [5] *Cr.*, *Cryphalus*; *D.*, *Dendroctonus*; *Dr.*, *Dryocoetes*; *I.*, *Ips*; *X.*, *Xylosandrus*. [6] ITS, the internal transcribed spacer regions 1 and 2 of the nuclear ribosomal DNA operon, including the 5.8S region; Tub2, the β-tubulin gene region (Tub2).

Maximum parsimony analyses were performed by using PAUP v. 4.0b10 [44], and the gaps were treated as a fifth base. Branch node confidence was estimated by using 1000 bootstrap replicates. The 50% majority of clades compatible in the bootstrap consensus tree was retained. The analytical settings were as follows: tree bisection reconnection branch swapping, starting tree obtained via stepwise addition, steepest descent not in effect, and MulTrees effective. For BI analyses, jModelTest v. 2.1.7 [47] was used to establish the best-fit substitution models for each dataset. Bayesian inference analyses were performed with MrBayes v. 3.1.2 [48], using four Markov chain Monte Carlo (MCMC) chains, and

chains were run simultaneously from a random starting tree for 5,000,000 generations to calculate posterior probabilities. Trees were sampled per 100 generations. The first 25% of trees sampled were set as burn-in values, and the remaining trees were used to calculate posterior probabilities. The final alignments and retrieved topologies were deposited in TreeBASE (No. 24415). Phylogenetic trees were edited by using FigTree v. 1.4.3 http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 20 November 2021) and Adobe Illustrator CS6.

2.5. Pathogenicity Tests

The type strain CFCC52689 was selected for in vivo pathogenicity tests in the field. The pathogenicity test was conducted on five-year-old healthy trees of *Larix olgensis* at Dagujia Forest Farm, Liaoning province, China. The tree height was approximately 2.5 m, and the ground diameter was approximately 3 cm. One wound was made on the trunk 30 cm from the ground, using a cork borer (6 mm diameter), and a 6 mm–diameter MEA plug was taken from the margins of an actively growing fungal colony and placed on the freshly wounded surface. For the control treatment, a sterile MEA plug was used. In total, three trees were inoculated with fungus, and three were inoculated with an MEA plug. The inoculated area was covered with Parafilm and wrapped with sticky tape. Inoculations were conducted on 11 July 2019. Two months later, the outer bark near the inoculated area was removed with a scalpel, and the lengths and widths of the lesions were measured and recorded on 11 September 2019. We used SPSS v. 10.0.1 (IBM Corp., Armonk, NY, USA) to analyze the differences in these lesions, using a one-way analysis of variance (ANOVA). Tissues at the margin of the lesions were collected to isolate and identify fungi to support Koch's postulates.

3. Results

3.1. Phylogenetics

For the phylogenetic inference of *Ceratocystiopsis*, ITS, Tub2, and combined (ITS + Tub2) datasets were constructed. For the combined dataset, the *p*-value of the homogeneity test is 0.029 (>0.01). The best models for the three datasets were estimated and applied in the BI as GTR+I+G (ITS, Tub2, and combined datasets). Alignments for the ITS and Tub2 datasets contained 679 and 550 characters (including gaps), respectively. The combined datasets included 31 sequences, representing 19 taxa with 1229 positions, including gaps. Our strains formed a separate branch with high node supports and were separated from a branch containing multiple species (*C. minima*, *C. minuta*, *C. weihaiensis*, and *Ceratocystiopsis* sp. 2) based on the phylogenetic trees of the combined datasets (Figure 1) and ITS datasets (Supplementary Materials Figure S1). In the phylogenetic analyses based on the individual Tub2 datasets, our strains clustered within a separate lineage with good supports (Supplementary Materials Figure S2).

3.2. Taxonomy

Ceratocystiopsis subelongati Z. Wang and Q. Lu, sp. nov. (Figure 2) MycoBank: MB 841972.

Etymology: The name is based on the vector (*Ips subelongatus*) from which this fungus was associated.

The sexual state was not observed. The asexual state is hyalorhinocladiella- to sporothrix-like. Sporothrix- to hyalorhinocladiella-like: Conidiophores mononematous, simple, upright or flexuous, arising from vegetative hyphae. Conidiogenous cells hyaline, blastic, not denticulate or occasionally denticulate, sometimes arising directly from hyphae, (12.7–) 15.6–31.8 (−48.0) × (1.2–) 1.3–1.7 (−2.0) µm. Conidia hyaline, smooth, clavate to ovate, and aseptate, (3.0–) 3.3–4.4 (−5.1) × (2.0–) 2.1–2.6 (−3.0) µm.

Culture characteristics: Colonies on 2% MEA initially hyaline, later becoming pure white, aerial mycelium sparse, and the colony margin thins radially, reaching 52 mm in

diameter in 22 days, at 25 °C. The optimal temperature for growth was 30 °C, and no growth was observed at 5 or 40 °C.

Habitat: Mixed forest of *Pinus sylvestris* var. *mongolica* and *Larix gmelinii*.

Host tree: *Pinus sylvestris* var. *mongolica*.

Distribution: Inner Mongolia, China.

Type. CHINA, Inner Mongolia, Hulunbuir City, Hailar national forest park (43°45′16″ N, 125°27′48″ E), from *Ips subelongatus* infesting *Pinus sylvestris* var. *mongolica*, August 2010, Z. Wang and Q. Lu, holotype CXY2015, ex-holotype CFCC52689.

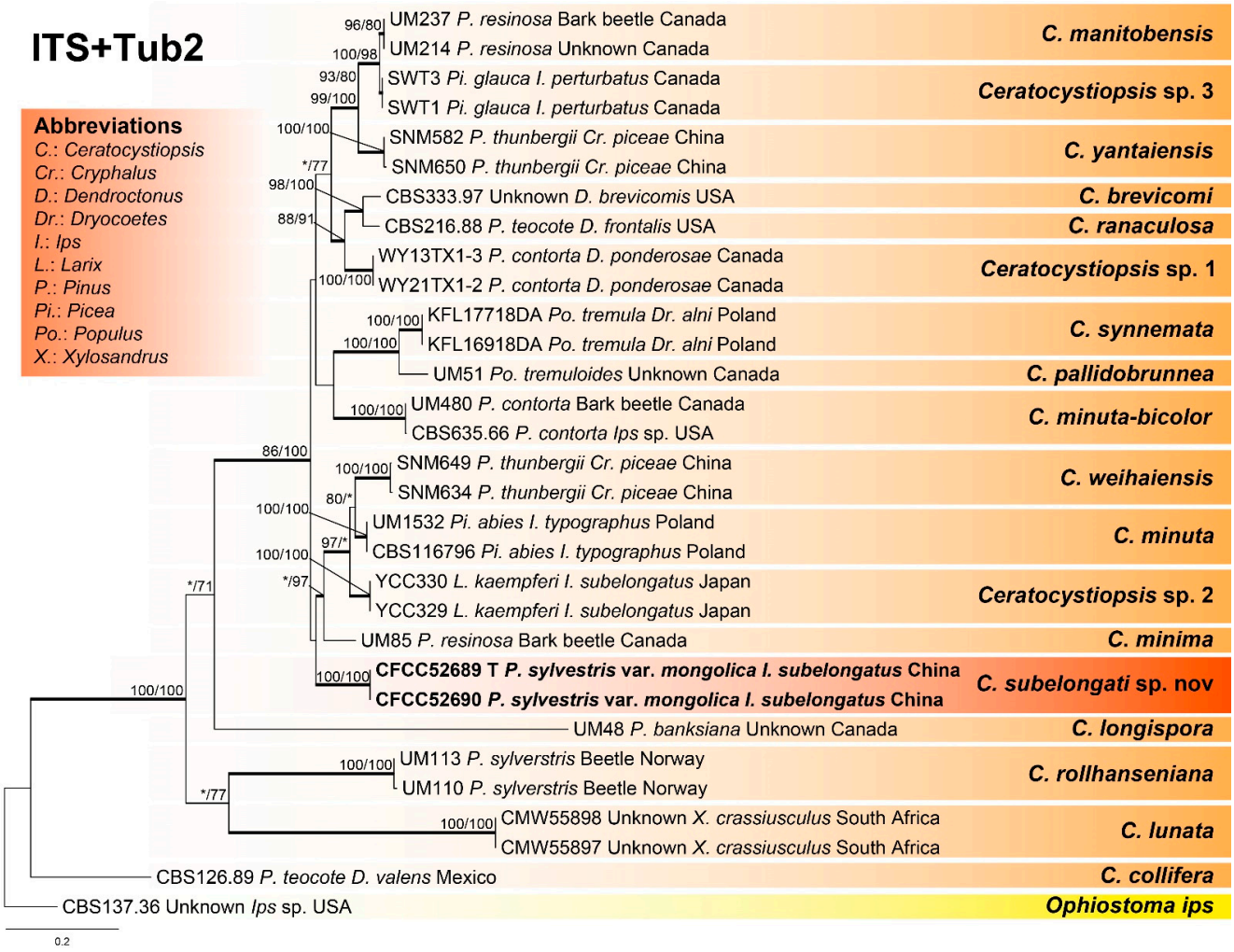

Figure 1. Phylogram of *Ceratocystiopsis* spp. based on combined (ITS + Tub2) sequence data. Bold branches indicate posterior probability values ≥ 0.9. Bootstrap values of ML/MP ≥ 70% are recorded at nodes. T = ex-type isolates. * Bootstrap values < 70%.

Notes: *Ceratocystiopsis subelongati* is characterized by a hyalorhinocladiella- to sporothrix-like asexual morph (Figure 2)—different from its sister taxon, *C. minima* (Figure 1 and Supplementary Materials Figure S1), which develops a hyalorhinocladiella-like asexual morph [49]. Different shapes of conidia are produced in *C. subelongati* (clavate to ovate) and *C. minima* (ellipsoid, cylindrical, clavate, or oblong). Although the phylogenetic relationships between *C. subelongati* and *C. minima* are very close, the hosts and distributions of the two differ markedly, with the latter being isolated from *Pinus banksiana* in Canada [49]. *Ceratocystiopsis minuta* was ever isolated from *I. subelongatus* infesting *Larix kaempferi* in Japan [50]; however, both species are distantly related in phylogenetic analyses (Figure 1 and Supplementary Materials Figures S1 and S2). It differs in hosts from *C. subelongati* (*L. kaempferi* vs. *P. sylvestris* var. *mongolica*), as well as in the shapes of the conidia (ellipsoidal

or cylindrical; oblong or ovoid vs. clavate to ovate), and optimal temperature (20 and 22 °C vs. 30 °C) [49].

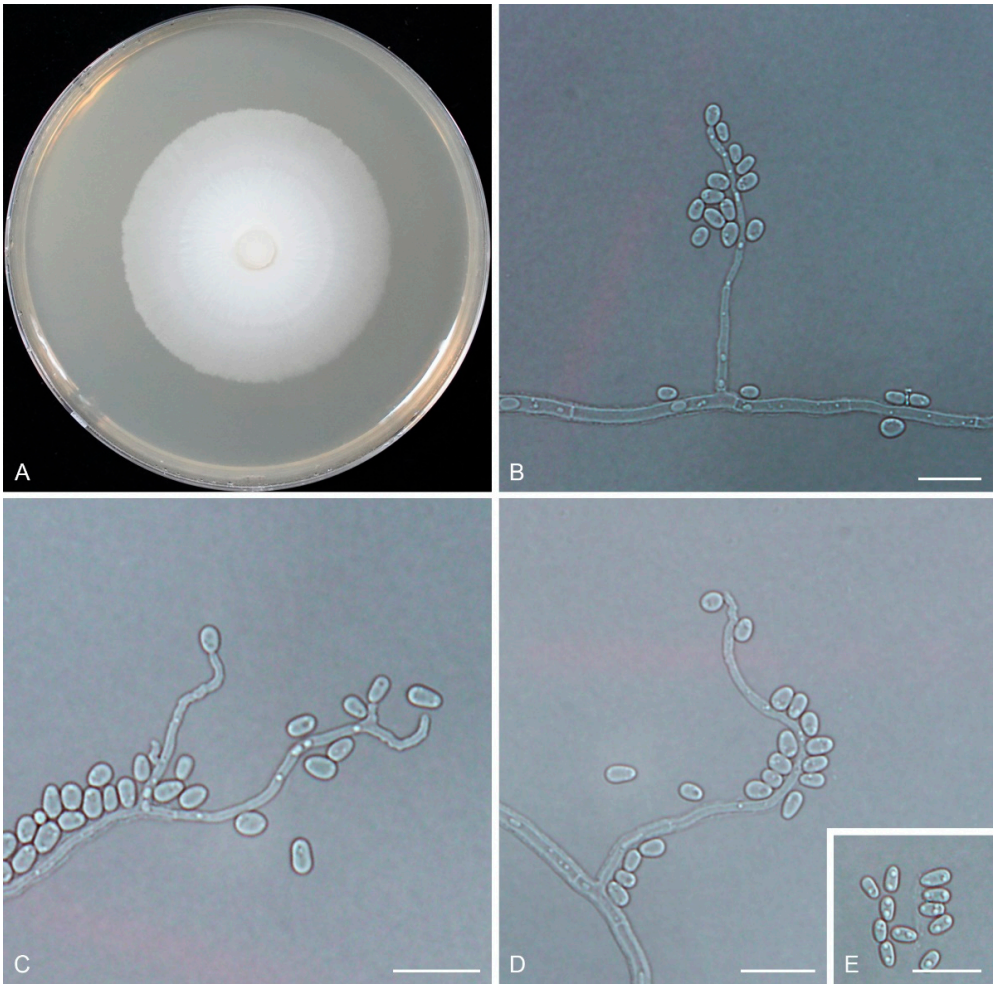

Figure 2. Morphological characteristics of *Ceratocystiopsis subelongati*: (**A**) 22-day-old culture on MEA, (**B**–**D**) conidiogenous cells of sporothrix- to hyalorhinocladiella-like asexual morph and conidia, and (**E**) conidia. Scale bars: (**B**–**D**) = 10 µm.

3.3. Pathogenicity

Two months after inoculation, although none of the three inoculated larch trees showed visual symptoms, 13.33 ± 0.58 mm × 14.0 ± 2.00 mm lesions were caused by *C. subelongati* under the bark in and around the site of inoculation, which was significantly different from the control (Table 2 and Supplementary Materials Figure S3). The inoculated fungus was easily re-isolated from the necrotic lesions, but it was not isolated from healthy tissue or control treatments inoculated with MEA plugs.

Table 2. Lesions observed in the inner bark of *Larix olgensis* two months after inoculation with *Ceratocystiopsis subelongati*.

Strain No.	Length (mm)	Wide (mm)
CFCC52689	13.33 ± 0.58	14.0 ± 2.00
Control	11.00 ± 0.82	11.3 ± 1.26
p-value	0.009	0.074

4. Discussion

In this study, *Ceratocystiopsis subelongati* was accurately identified and described based on the phylogenetic inference of multi-gene DNA sequences and morphological characteristics. Pathogenicity tests showed that this fungus was pathogenic to five-year-old *L. olgensis* in the field. Due to the lack of reference sequences of related species, this species was represented as *C.* cf. *pallidobrunnea* in a previous study by Wang et al. [20]. Since then, the availability and publication of ITS and Tub2 of *C. pallidobrunnea* [17] have made it possible to compare. Phylogenetic analysis based on ITS and Tub2 datasets showed that *C. subelongati* and *C. pallidobrunnea* were far related, which contradicted the results based on LSU dataset [20]. This is probably because the LSU sequence of *C. pallidobrunnea* available is incomplete—only 562 bp (GenBank accession number: EU913682)—and a large number of gaps after alignment may bring errors to phylogenetic analysis. For *Ceratocystiopsis*, DNA sequences available for phylogenetic analysis in public databases, such as GenBank, remain limited. For example, only incomplete LSU sequences were available for *C. concentrica* and *C. parva*. Therefore, there is an urgent need for multi-gene sequencing of type strains of different species in this genus to re-evaluate their taxonomic status. In addition, we sequenced the TEF1-α gene region of *C. subelongati* and presented it in the Supplementary Materials (named TEF1-α sequences).

Ips subelongatus is mainly distributed in China, Japan, Mongolia, North Korea, Russia, and South Korea [51]. In China, it has been recorded in ten northern provinces [51,52]. Twenty-two species of ophiostomatoid fungi have been so far reported to associate with this beetle, but the investigation was only based on four northeastern provinces [20,36,53–56]. There are likely to be more ophiostomatoid fungi associated with *I. subelongatus* awaiting discovery, especially in the vast coniferous forests of Northwest China.

To date, six species of ophiostomatoid fungi associated with *I. subelongatus* have been subjected to pathogenicity tests in China. *Endoconidiophora fujiensis* can cause lesions >70 cm in length in *L. kaempferi* over 2 months but are weakly virulent in the three local larches (*L. principis-rupprechtii*, *L. gmelinii*, and *L. olgensis*) [20]. Consistently, under artificial inoculation, the fungus demonstrated the ability to kill 30-year-old Japanese larch trees (*L. kaempferi*) within 3.5 months in Japan [50,57]. *Endoconidiophora fujiensis* seems to be the most threatening pathogenic fungi associated with *I. subelongatus* to its host conifers. Three *Leptographium* species (*L. innermongolicum*, *L. taigense*, and *L. zhangii*) and one *Ophiostoma* species (*O. olgensis*) have also been shown to be weakly virulent in different larch trees [55, 56]. In this study, *C. subelongati* was weakly pathogenic to larch trees, either.

The ophiostomatoid fungi are well-known as symbionts of numerous bark beetles, playing roles of synergistically overcoming host defenses, nutrition suppliers, and regulating beetle behavior. Most *Ceratocystiopsis* species are recorded to associate with bark beetles infecting conifers. Among them, only *C. brevicomi* has been shown to be mutualistic with *Dendroctonus brevicomis* [58] and is involved in cascading speciation among *P. ponderosa–D. brevicomis* fungal mutualists (*C. brevicomi* and *Entomocorticium* sp. B) [59]. Thus, the role of other *Ceratocystiopsis* species in the evolution and lifecycle of bark beetles, as well as their pathogenicity in plants, remains to be studied.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12121795/s1. Figure S1: Phylogram of *Ceratocystiopsis* spp. based on based on ITS sequence data. Bold branches indicate posterior probability values ≥ 0.9. Bootstrap values of ML/MP ≥ 70% are recorded at nodes. T = ex-type isolates. * Bootstrap values < 70%. Figure S2: Phylogram of *Ceratocystiopsis* spp. based on Tub2 sequence data. Bold branches indicate posterior probability values ≥ 0.9. Bootstrap values of ML/MP ≥ 70% are recorded at nodes. T = ex-type isolates. * Bootstrap values < 70%. Figure S3: Symptoms developed in the trunk of *Larix olgensis* inoculated with *Ceratocystiopsis subelongati* two months after inoculation. (A) Inoculation of *C. subelongati* and (B) control.

Author Contributions: Conceptualization, Z.W., Z.L. and Q.L.; methodology, Z.W., Y.L. and Q.L.; software, Z.W. and Y.L.; validation, Z.W., Z.L. and Q.L.; formal analysis, Z.W., C.L. and Y.L.; investigation, Z.W., Y.L. and L.L.; resources, Z.W., Z.L. and Q.L.; data curation, Z.W., Y.L. and Q.L.; writing—original draft preparation, Z.W.; writing—review and editing, Q.L.; visualization, Z.W.; supervision, Q.L.; project administration, Q.L.; funding acquisition, Q.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are openly available from GenBank.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. De Beer, Z.W.; Seifert, K.A.; Wingfield, M.J. The ophiostomatoid fungi: Their dual position in the Sordariomycetes. *CBS Biodivers. Ser.* **2013**, *12*, 1–19.
2. Wingfield, M.J.; Barnes, I.; De Beer, Z.W.; Roux, J.; Wingfield, B.D.; Taerum, S.J. Novel associations between ophiostomatoid fungi, insects and tree hosts: Current status—future prospects. *Biol. Invasions* **2017**, *19*, 3215–3228. [CrossRef]
3. Malloch, D.W.; Blackwell, M. Dispersal biology of the ophiostomatoid fungi. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*; Wingfield, M.J., Seifert, K.A., Webber, J., Eds.; APS Press: St. Paul, MN, USA, 1993; pp. 195–206.
4. Kirisits, T. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In *Bark and Wood Boring Insects in Living Trees in Europe*; Lieutier, F., Day, K.R., Battisti, A., Grégoire, J.C., Evans, H., Eds.; A Synthesis: Dordrecht, The Netherlands, 2004; pp. 185–223.
5. Biedermann, P.H.; Vega, F.E. Ecology and Evolution of Insect–Fungus Mutualisms. *Annu. Rev. Èntomol.* **2020**, *65*, 431–455. [CrossRef] [PubMed]
6. Six, D.L. The Bark Beetle Holobiont: Why Microbes Matter. *J. Chem. Ecol.* **2013**, *39*, 989–1002. [CrossRef]
7. Bracewell, R.R.; Six, D.L. Experimental evidence of bark beetle adaptation to a fungal symbiont. *Ecol. Evol.* **2015**, *5*, 5109–5119. [CrossRef]
8. Zhao, T.; Axelsson, K.; Krokene, P.; Borg-Karlson, A.K. Fungal Symbionts of the Spruce Bark Beetle Synthesize the Beetle Aggregation Pheromone 2-Methyl-3-buten-2-ol. *J. Chem. Ecol.* **2015**, *41*, 848–852. [CrossRef] [PubMed]
9. Zhao, T.; Ganji, S.; Schiebe, C.; Bohman, B.; Weinstein, P.; Krokene, P.; Borg-Karlson, A.; Unelius, C.R. Convergent evolution of semiochemicals across Kingdoms: Bark beetles and their fungal symbionts. *ISME J.* **2019**, *13*, 1535–1545. [CrossRef] [PubMed]
10. Cale, J.A.; Ding, R.; Wang, F.; Rajabzadeh, R.; Erbilgin, N. Ophiostomatoid fungi can emit the bark beetle pheromone verbenone and other semiochemicals in media amended with various pine chemicals and beetle-released compounds. *Fungal Ecol.* **2019**, *39*, 285–295. [CrossRef]
11. Davis, T.S.; Stewart, J.E.; Mann, A.; Bradley, C.; Hofstetter, R.W. Evidence for multiple ecological roles of *Leptographium abietinum*, a symbiotic fungus associated with the North American spruce beetle. *Fungal Ecol.* **2019**, *38*, 62–70. [CrossRef]
12. Kandasamy, D.; Gershenzon, J.; Andersson, M.N.; Hammerbacher, A. Volatile organic compounds influence the interaction of the Eurasian spruce bark beetle (*Ips typographus*) with its fungal symbionts. *ISME J.* **2019**, *13*, 1788–1800. [CrossRef] [PubMed]
13. Guevara-Rozo, S.; Hussain, A.; Cale, J.A.; Klutsch, J.G.; Rajabzadeh, R.; Erbilgin, N. Nitrogen and Ergosterol Concentrations Varied in Live Jack Pine Phloem Following Inoculations with Fungal Associates of Mountain Pine Beetle. *Front. Microbiol.* **2020**, *11*, 1703. [CrossRef] [PubMed]
14. Upadhyay, H.P.; Kendrick, W.B. Prodromus for a Revision of Ceratocystis (*Microascales, Ascomycetes*) and its Conidial States. *Mycologia* **1975**, *67*, 798–805. [CrossRef]
15. Hausner, G.; Reid, J.; Klassen, G. Ceratocystiopsis: A reappraisal based on molecular criteria. *Mycol. Res.* **1993**, *97*, 625–633. [CrossRef]
16. Zipfel, R.D.; de Beer, Z.W.; Jacobs, K.R.; Wingfield, B.D.; Wingfield, M.J. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Stud. Mycol.* **2006**, *55*, 75–97. [CrossRef]
17. Strzałka, B.; Jankowiak, R.; Bilański, P.; Patel, N.; Hausner, G.; Linnakoski, R.; Solheim, H. Two new species of *Ophiostomatales* (*Sordariomycetes*) associated with the bark beetle *Dryocoetes alni* from Poland. *MycoKeys* **2020**, *68*, 23–48. [CrossRef] [PubMed]
18. Nel, W.J.; Wingfield, M.J.; de Beer, Z.W.; Duong, T.A. Ophiostomatalean fungi associated with wood boring beetles in South Africa including two new species. *Antonie van Leeuwenhoek* **2021**, *114*, 667–686. [CrossRef]
19. Chang, R.; Zhang, X.; Si, H.; Zhao, G.; Yuan, X.; Liu, T.; Bose, T.; Dai, M. Ophiostomatoid species associated with pine trees (*Pinus* spp.) infested by *Cryphalus piceae* from eastern China, including five new species. *MycoKeys* **2021**, *83*, 181–208. [CrossRef]
20. Wang, Z.; Liu, Y.; Wang, H.; Meng, X.; Liu, X.; Decock, C.; Zhang, X.; Lu, Q. Ophiostomatoid fungi associated with *Ips subelongatus*, including eight new species from northeastern China. *IMA Fungus* **2020**, *11*, 3. [CrossRef]

21. Six, D.L.; Wingfield, M.J. The Role of Phytopathogenicity in Bark Beetle–Fungus Symbioses: A Challenge to the Classic Paradigm. *Annu. Rev. Entomol.* **2011**, *56*, 255–272. [CrossRef] [PubMed]
22. Eckhardt, L.G. Blackstain root disease and other *Leptographium diseases*. In *Infectious Forest Diseases*; Gonthier, P., Nicolotti, G., Eds.; CAB International: Wallingford, CT, USA, 2013; pp. 283–297.
23. Harrington, T.C. Ceratocystis Diseases. In *Infectious Forest Diseases*; Gonthier, P., Nicolotti, G., Eds.; CAB International: Wallingford, CT, USA, 2013; pp. 230–255.
24. Kirisits, T. Dutch Elm Disease and Other Ophiostoma Diseases. In *Infectious Forest Diseases*; Gonthier, P., Nicolotti, G., Eds.; CAB International: Wallingford, CT, USA, 2013; pp. 256–282.
25. Barnes, I.; Fourie, A.; Wingfield, M.; Harrington, T.; McNew, D.; Sugiyama, L.; Luiz, B.; Heller, W.; Keith, L. New *Ceratocystis* species associated with rapid death of *Metrosideros polymorpha* in Hawaii. *Persoonia* **2018**, *40*, 154–181. [CrossRef] [PubMed]
26. Brasier, C.M. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **1991**, *115*, 151–161. [CrossRef]
27. Harrington, T.C.; Cobb, F.W. *Leptographium* Root Diseases on Conifers. *Mycologia* **1989**, *81*, 330. [CrossRef]
28. Fraedrich, S.W.; Harrington, T.C.; Rabaglia, R.J.; Ulyshen, M.D.; Mayfield, A.E.; Hanula, J.L.; Eickwort, J.M.; Miller, D.R. A Fungal Symbiont of the Redbay Ambrosia Beetle Causes a Lethal Wilt in Redbay and Other *Lauraceae* in the Southeastern United States. *Plant Dis.* **2008**, *92*, 215–224. [CrossRef]
29. Chang, R.; Duong, T.; Taerum, S.; Wingfield, M.; Zhou, X.; Yin, M.; de Beer, Z.W. Ophiostomatoid fungi associated with the spruce bark beetle Ips typographus, including 11 new species from China. *Persoonia* **2019**, *42*, 50–74. [CrossRef]
30. Yin, M.; Wingfield, M.J.; Zhou, X.; Linnakoski, R.; De Beer, Z.W. Taxonomy and phylogeny of the *Leptographium olivaceum* complex (*Ophiostomatales*, *Ascomycota*), including descriptions of six new species from China and Europe. *MycoKeys* **2019**, *60*, 93–123. [CrossRef]
31. Yin, M.; Wingfield, M.J.; Zhou, X.; De Beer, Z.W. Phylogenetic re-evaluation of the *Grosmannia penicillata* complex (*Ascomycota*, *Ophiostomatales*), with the description of five new species from China and USA. *Fungal Biol.* **2019**, *124*, 110–124. [CrossRef]
32. Wang, H.M.; Wang, Z.; Liu, F.; Wu, C.X.; Zhang, S.F.; Kong, X.B.; Decock, C.; Lu, Q.; Zhang, Z. Differential patterns of ophiostomatoid fungal communities associated with three sympatric *Tomicus* species infesting pines in south-western China, with a description of four new species. *MycoKeys* **2019**, *50*, 93–133. [CrossRef]
33. Wang, Z.; Liu, Y.; Wang, T.; Decock, C.; Chu, B.; Zheng, Q.; Lu, Q.; Zhang, X. *Grosmannia tibetensis*, a new ophiostomatoid fungus associated with *Orthotomicus* sp. (*Coleoptera*) in Tibetan subalpine forests. *Mycoscience* **2020**, *61*, 282–292. [CrossRef]
34. Wang, Z.; Zhou, Q.; Zheng, G.; Fang, J.; Han, F.; Zhang, X.; Lu, Q. Abundance and diversity of ophiostomatoid fungi associated with the Great Spruce Bark Beetle (*Dendroctonus micans*) in the Northeastern Qinghai-Tibet Plateau. *Front. Microbiol.* **2021**, *12*, 3082. [CrossRef] [PubMed]
35. Marincowitz, S.; Duong, T.; Taerum, S.; De Beer, Z.; Wingfield, M. Fungal associates of an invasive pine-infesting bark beetle, *Dendroctonus valens*, including seven new Ophiostomatalean fungi. *Persoonia* **2020**, *45*, 177–195. [CrossRef]
36. Chang, R.; Wingfield, M.J.; Marincowitz, S.; de Beer, Z.W.; Zhou, X.; Duong, T.A. Ophiostomatoid fungi including a new species associated with Asian larch bark beetle *Ips subelongatus*, in Heilongjiang (Northeast China). *Fungal Syst. Evol.* **2021**, *8*, 155–161. [CrossRef]
37. Pan, Y.; Lu, J.; Zhou, X.D.; Yu, Z.F.; Chen, P.; Wang, J.; Ye, H. Two new species of *Leptographium* associated with *Tomicus* spp. infesting *Pinus* spp. in Southwestern China. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 4798–4807. [CrossRef]
38. Rayner, R.W. A mycological colour chart. In *Commonwealth Mycological Institute and British Mycological Society*; Commonwealth Mycological Institute & British Mycological Society: Kew, UK, 1970.
39. White, T.J.; Bruns, T.; Lee, S.J.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: A guide to methods and applications. *PCR Protoc. A Guide Methods Appl.* **1990**, *18*, 315–322. [CrossRef]
40. Glass, N.L.; Donaldson, G.C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Appl. Environ. Microbiol.* **1995**, *61*, 1323–1330. [CrossRef]
41. Jacobs, K.; Bergdahl, D.R.; Wingfield, M.J.; Halik, S.; Seifert, K.A.; Bright, D.E.; Wingfield, B.D. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycol. Res.* **2004**, *108*, 411–418. [CrossRef]
42. Katoh, K.; Rozewicki, J.; Yamada, K.D. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* **2019**, *20*, 1160–1166. [CrossRef]
43. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
44. Swofford, D.L. *Paup *: Phylogenetic Analyses Using Parsimony (* and Other Methods) Version 4.0b10*; Sinauer Associates: Sunderland, MA, USA, 2003.
45. Stamatakis, A. RaxML Version 8: A tool phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **2014**, *30*, 1312–1313. [CrossRef]
46. Stamatakis, A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **2006**, *22*, 2688–2690. [CrossRef]
47. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat Methods* **2012**, *9*, 772. [CrossRef]

48. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **2003**, *19*, 1572–1574. [CrossRef] [PubMed]
49. Upadhyay, H.P. *A Monograph of Ceratocystis and Ceratocystiopsis*; University of Georgia Press: Athens, GA, USA, 1981.
50. Yamaoka, Y.; Wingfield, M.J.; Ohsawa, M.; Kuroda, Y. Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch. *Mycoscience* **1998**, *39*, e367–e378. [CrossRef]
51. Cognato, A.I. Biology, systematics, and evolution of *Ips*. In *Bark Beetles*; Vega, F.E., Hofstetter, R.W., Eds.; Academic Press: San Diego, CA, USA, 2015; pp. 351–370. [CrossRef]
52. Yin, H.; Huang, F.; Li, Z. Coleoptera: Scolytidae. In *Economic insect fauna of China (Fasc. 29)*; Science press: Beijing, China, 1984; pp. 126–136.
53. Paciura, D.; de Beer, Z.W.; Jacobs, K.; Zhou, X.D.; Ye, H.; Wingfield, M.J. Eight new *Leptographium* species associated with tree-infesting bark beetles in China. *Persoonia* **2010**, *25*, 94–108. [CrossRef]
54. Liu, X.; Lu, Q.; Meng, X.; Jiao, X.; Liang, J.; Zhang, X. Identification and phylogeny of *Graphium* spp. (*Microascales*: *Graphiaceae*) associated with *Ips subelongatus* (*Coleoptera*: *Scolytidae*) in China. *Sci. Silvae Sin.* **2015**, *52*, 76–86. [CrossRef]
55. Wang, H.M.; Lu, Q.; Meng, X.J.; Liu, X.W.; Decock, C.; Zhang, X.Y. *Ophiostoma olgensis*, a new species associated with *Larix* spp. and *Ips subelongatus* in northern China. *Phytotax* **2016**, *282*, 282–290. [CrossRef]
56. Liu, X.W.; Wang, H.M.; Lu, Q.; Decock, C.; Li, Y.X.; Zhang, X.Y. Taxonomy and pathogenicity of *Leptographium*, species associated with *Ips subelongatus*, infestations of *Larix* spp. in northern China, including two new species. *Mycol. Prog.* **2017**, *16*, 1–13. [CrossRef]
57. Yamaoka, Y. Taxonomy and pathogenicity of ophiostomatoid fungi associated with bark beetles infesting conifers in Japan, with special reference to those related to subalpine conifers. *Mycoscience* **2017**, *58*, 221–235. [CrossRef]
58. Dysthe, J.C.; Bracewell, R.; Six, D.L. Temperature effects on growth of fungal symbionts of the western pine beetle, *Dendroctonus brevicomis*. *Fungal Ecol.* **2015**, *17*, 62–68. [CrossRef]
59. Bracewell, R.R.; Vanderpool, D.; Good, J.M.; Six, D.L. Cascading speciation among mutualists and antagonists in a tree–beetle–fungi interaction. *Proc. R. Soc. B* **2018**, *285*, 20180694. [CrossRef] [PubMed]