Ophiostomatoid Fungi Associated with *Cryphalus Piceae* in Shandong province in eastern China

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Abstract

Cryphalus piceae parasitizes various economically important conifers. Similar to other bark beetles, *C. picea* vectors an assortment of fungi and nematodes. Previously, several ophiostomatoid fungi were isolated from *C. piceae* in Poland and Japan. In the present study, we explored the diversity of ophiostomatoid fungi associated with *C. piceae* infesting pines in the Shandong Province of China. We isolated ophiostomatoid fungi from both galleries and beetles collected from our study sites. These fungal isolates were identified using both molecular and morphological data. Through this study, we recovered 176 isolates of ophiostomatoid fungi representing at least seven species. *Ophiostoma ips* was the most frequently isolated species. Analyses of molecular and morphological data indicated four of the ophiostomatoid fungal species recovered in this study were previously undescribed. Hereby, we described these species as *Ceratocystiopsis yantaiensis* sp. nov., *C. weihaiensis* sp. nov., *Graphilbum translucens* sp. nov. and *Sporothrix villosa* sp. nov. A majority of the ophiostomatoid fungi recovered in this study were novel species. This suggests that the forests in China harbour an assortment of undescribed ophiostomatoid fungi yet to be discovered.

Introduction

Globally, *Bursaphelenchus xylophilus* (pinewood nematode) infects a variety of pine species causing the 'pine wilt disease' that kills affected trees within a few weeks to months (Futai, 2013; Donald et al., 2016). In China, Japan and Korea this nematode is predominantly transmitted by *Monochamus alternatus* (*Cerambycidae*), the Japanese pine sawyer beetle (Mamiya & Enda, 1972; Morimoto & Iwasaki, 1972; Li et al., 2007; Donald et al., 2016). Other insect vectors of this nematode are *Dendroctonus frontalis* (Scolytidae), and *Acanthocinus griseus* (Cerambycidae) (Ryss et al., 2005). Besides the nematodes, various other microbes are also known to associate with these beetles such as ophiostomatoid fungi.

Ophiostomatoid fungi often form a symbiotic association with bark and ambrosia beetles who assist in the dispersal of their inocula (Klepzig & Six, 2004). For example, *Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A remain associated with the mycangium of *D. frontalis* whereas *Ophiostoma minus* is carried phoretically on the exoskeleton (Hofstetter et al., 2015). Moreover, an ophiostomatoid fungus can symbiotically associate with multiple beetle species. Recently, six ophiostomatoid fungi were isolated from *M. alternatus* in China (Zhao et al., 2014; Wang et al., 2018). Among them, *Ophiostoma ips* were previously isolated from *B. xylophilus* and *M. alternatus* from North America and Korea, respectively (Wingfield, 1987; Suh et al., 2013).

Ophiostomatoid fungi are a polyphyletic group of fungi that includes several genera in the orders *Microascales* and *Ophiostomatales*. These fungi are characterized by spores produced in sticky droplets that facilitate dispersal by bark beetles and mites (De Beer et al., 2013). *Microascales* includes three ophiostomatoid fungal families. These are *Ceratocystidaceae* (11 genera), *Gondwanamycetaceae* (2 genera), and *Graphiaceae* (1 genus) (De Beer et al., 2013). *Ophiostomatales* is represented by a single family, *Ophiostomataceae* (11 genera) (De Beer et al., 2016a; De Beer et al., 2016b; Van der Linde et al., 2016; Bateman et al., 2017; Poinar & Vega, 2018). Initially, De Beer and Wingfield (2013) recognized 18 species complexes in the order *Ophiostomatales*. Later, '*S. schenckii* – *O. stenoceras*' species complex was elevated to genus *Sporothrix.* Thereafter, the genus was subsequently divided into six species complexes (De Beer et al., 2016a).

In Europe and Asia, *Cryphalus piceae* parasitizes various species of *Abies*, *Pinus*, *Picea* and *Larix* (Jankowiak & Kolarik, 2010). This bark beetle predominantly affects stressed trees (Michalski & Mazur, 1999), but can also infect healthy ones (Justesen et al., 2020). Previously, several fungal species were isolated from *C. piceae*. This included an assortment of ophiostomatoid fungi and *Geosmithia* from Poland (Jankowiak & Kolarik, 2010; Jankowiak & Bilanski, 2018) and Japan (Ohtaka et al., 2002a; Ohtaka et al., 2002b). In the present study, we explored the diversity of ophiostomatoid fungi associated with *C. piecea* from the pine plantations located in the Shandong Province of China.

Material And Method

Collection of beetles and isolation of fungi

From September 2019 – August 2020 multiple surveys were conducted in pine plantations located near Weihai (N37°30'07" E12°07'24"), Qingdao (N36°15'26" E12°38'07") and Yantai (N37°15'38" E12°44'39"), Shandong Province, China. Beetle galleries from affected trees were collected in polyethene bags. Whereas, adult beetles from these sampled galleries were collected in 2 ml sterile collection tubes. Both galleries and beetles were stored at 4 °C until the isolation of fungi.

Isolation of fungi was done using the method suggested by Chang et al. (2019). Fungal mycelium and/or spore masses from *C. piecea* galleries were transferred onto 2 % malt extract agar (MEA) medium amended 0.05 % streptomycin. In cases where no mycelia were visible, galleries were incubated in moist chambers at 25°C in darkness for 4–6 weeks. Post incubation, conidia with spore masses emerging from these incubated galleries were transferred onto MEA amended with streptomycin. For isolating fungi from the beetles, adult *C. piecea* were crushed on the surface of MEA amended streptomycin. In order to purify the fungal isolates, hyphal tips from colonies were transferred onto fresh MEA plates.

All fungal isolates were submitted to the microbial culture collection of Shandong Normal University, Jinan, Shandong, China (SNM; for accession numbers see Table 1). Ex-holotypes culture of ophiostomatoid fungi described in this study were deposited in the China General Microbiological Culture Collection Center (CGMCC), Beijing, China. Holotype specimens (dry culture) were deposited in the Herbarium Mycologicum, Academiae Sinicae (HMAS), Beijing, China.

Table 1
Isolates of ophiostomatoid fungi obtained from *Cryphalus piceae* and used in this study.

Taxon	Species	Isolate[1]	CGMCC[2]	Location	B/G[3]	ITS[4]	BT[5]	EF[6]	CAL[7]
1	*Ceratocystiopsis yantaiensis* sp. nov.	SNM582		Yantai	G	MW989410	MZ019522	-	-
		SNM650^T	3.20247	Yantai	G	MW989411	MZ019523	-	-
2	*Ceratocystiopsis weihaiensis* sp. nov.	SNM634		Weihai	G	MW989412	MZ019524	-	-
		SNM649^T	3.20246	Weihai	G	MW989413	MZ019525	-	-
3	*Graphilbum traslucens* sp. nov.	SNM101		Weihai	G	MW989414	MZ019526	MZ019544	-
		SNM104		Qingdao	G	MW989415	MZ019527	MZ019545	-
		SNM144^T	3.20263	Weihai	G	MW989416	MZ019528	MZ019546	-
4	*Graphilbum crescericum*	SNM100		Qingdao	G	MW989417	MZ019529	MZ019547	-
		SNM145		Weihai	B	MW989418	MZ019530	MZ019548	-
5	*Graphium pseudormiticum*	SNM159		Weihai	G	MW989419	-	MZ019549	-
6	*Ophiostoma ips*	SNM20		Weihai	G	MW989420	MZ019531	-	-
		SNM44		Weihai	G	MW989421	MZ019532	-	-
		SNM110		Weihai	G	MW989422	MZ019533	-	-
		SNM120		Weihai	G	MW989423	MZ019534	-	-
		SNM121		Weihai	G	MW989424	MZ019535	-	-
7	*Sporothrix villosa* sp. nov.	SNM162		Weihai	B	MW989425	MZ019536	-	MZ019540
		SNM182		Weihai	B	MW989426	MZ019537	-	MZ019541
		SNM185		Weihai	G	MW989427	MZ019538	-	MZ019542
		SNM188^T	3.20264	Weihai	B	MW989428	MZ019539		MZ019543

[1] The culture collection (SNM) of Shandong Normal University, Jinan, Shandong, China

[2] The China General Microbiological Culture Collection Center (CGMCC), Beijing, China

[3] B = Beetle; G = Gallery

[4] ITS = internal transcribed spacer regions 1 and 2 of the nuclear ribosomal DNA operon, including the 5.8S region

[5] BT = beta–tubulin

[6] EF = translation elongation factor 1–alpha

[7] CAL = Calmodulin

DNA extraction, PCR amplification and sequencing

All the isolates obtained in this study were initially grouped based on colony morphologies. For the purpose of preliminary identification, at least two representative isolates from each group were identified using the molecular technique. For the novel species described in the present study, all isolates were sequenced to confirm their identity.

The PrepMan ultra sample preparation reagent (Applied Biosystems, Foster City, CA) was used for extracting the total genomic DNA from five-day-old cultures, following the manufacturer's protocols. The complete ITS region and partial β-tubulin (BT), elongation factor 1-α (EF), and calmodulin (CAL) genes were amplified using primers ITS1F/ITS 4 (White et al., 1990; Gardes & Bruns, 1993), Bt2a (or T10)/Bt2b (Glass & Donaldson, 1995), EF2F/EF2R (Jacobs et al., 2004; Marincowitz et al., 2015), and CL2F/CL2R (Duong et al., 2012), respectively.

Each 25 µl PCR reaction included 12.5 µl 2 × Taq Master Mix (buffer, dNTPs and Taq; Vazyme Biotech Co., Ltd, China), 0.5 µl each of forward and reverse primers, 10.5 µl PCR grade water, and 1 µl of DNA template. PCR amplifications were conducted with an initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 60 sec; annealing temperature was 55 ºC for 60 sec for all primers; 72°C for 1 min; and final elongation at 72°C for 10 min.

All the PCR products were sequenced by the Sangon Biotech, Qingdao, Shandong Province, China. The resulting sequences were assembled using Geneious v. 7.1.4 (Biomatters, Auckland, New Zealand). The BLAST algorithm (Altschul et al. 1990) available through the NCBI GenBank was used for the preliminary identification of the sequences. All the sequences were submitted to GenBank and the accession numbers are listed in Table 1.

Phylogenetic analyses

For the purpose of phylogenetic analyses, separate datasets were prepared for all four gene regions (ITS, BT, EF and CAL). Each of these datasets included sequences generated in this study, and those that were retrieved from the GenBank (including the ex-type sequences). We recovered multiple isolates of the same species from *O. ips* and *S. gossypina* complex. Therefore, datasets for these two complexes included sequences from at least four representative isolates. The dataset was aligned using MAFFT v. 7 (Katoh & Standley, 2013). If needed, alignments were manually edited using MEGA v. 6.06 (Tamura et al., 2013). All aligned sequence datasets were deposited to TreeBase (Acc. No. 28127).

Programs used for Maximum likelihood (ML) and Bayesian inference (BI) analyses were accessed through the CIPRES Science Gateway v. 3.3 (Miller et al., 2010). For all datasets, jModelTest v. 2.1.6 (Darriba et al., 2012) was used for selecting appropriate substitution models. ML analyses were done through RaxML v. 8.2.4 (Stamatakis, 2014) using the GTR substitution model and 1000 bootstrap replicates. BI analyses were done using MrBayes v. 3.2.6 (Ronquist et al., 2012). Four MCMC chains were run from a random starting tree for five million generations and trees were sampled every 100th generation. One-fourth of the sampled trees were discarded as burn-in and the remaining trees were used for constructing majority rule consensus trees. MEGA-X was used for conducting maximum parsimony (MP) analyses (Kumar et al., 2018) where gaps were treated as a fifth character.

Growth and morphological studies

A representative isolate for each new fungal species identified through phylogenetic analyses was selected for morphological study. Isolates were initially sub-cultured on 2% MEA and incubated for seven days at 25°C in darkness. Thereafter, 5 mm agar plugs were placed at the centres of 90 mm Petri dishes and three replicate plates per isolate were incubated at 5, 10, 15, 20, 25, 30 and 35 ºC (± 0.5 ºC) in darkness. The colony diameter of each isolate was measured at an interval of two days up to the tenth day.

Microscopic structures of the ophiostomatoid fungi were measured and photographed using a Zeiss Axio Imager Z2 (CarlZeiss, Germany). Fifty measurements for each taxonomically informative structure were made, such as conidiophore and conidia.

Results

Collection of beetles and isolation of fungi

In the present study, 176 isolates of ophiostomatoid fungi were recovered. Among these, 148 were isolated from galleries whereas 28 from beetles. Based on the collection sites, 16 isolates were recovered from Yantai, 63 from Qingdao and 97 from Weihai.

Phylogenetic analyses

Preliminary identification of the ophiostomatoid fungi recovered in this study showed that the isolates resided in *Ceratocystiopsis* (4 isolates), *Graphilbum* (6 isolates), *Graphium* (1 isolate), *Ophiostoma* (141 isolates) and *Sporothrix* (24 isolates).

Species residing in *Ceratocystiopsis* were analyzed using ITS and BT gene regions. In the phylogenies of *Ceratocystiopsis*, four isolates of *Ceratocystiopsis* recovered in this study clustered into two distinct monophyletic clades (Fig. 1). Taxon 1 (two isolates) and Taxon 2 (two isolates) are sister species to *C. manitobensis* and *C. minuta*, respectively (Fig. 1).

Species residing in *Graphilbum* were analyzed using ITS, BT and EF gene regions. Phylogenetic analyses of six isolates clustered them into two distinct monophyletic clades (Fig. 2). Taxon 3 (four isolates) is closely related to *Gr. puerense* and *Gr. acuminatum* whereas Taxon 4 (two isolates) was *Gr. crescericum*.

The identity of the isolate residing in *Graphium* was confirmed using ITS and EF gene regions. Taxon 5 (one isolate) was identified as *G. pseudormiticum* (Fig. S1).

Species resided in *O. ipx* complex were analyzed using ITS and BT gene regions. In the ITS and BT trees, our isolates (Taxon 6, 141 isolates) formed monophyletic clades with *O. ips* (Fig. S2).

Isolates from the *S. gossypina* complex were analyzed using ITS, BT and CAL gene regions. Phylogenetic analyses showed those isolates (Taxon 7, 24 isolates) were closely related to two fungal isolates from China that were previously identified as *S. cf. abietina* (Fig. 3).

Taxonomy

Taxon 1

Ceratocystiopsis yantaiensis R.L. Chang & X.Y. Zhang, sp. nov.

— MycoBank MB839252; Fig. 4

Etymology

Name refers to Yantai City, where this fungus was isolated.

Diagnosis: *Ceratocystiopsis yantaiensis* is different from closely related species by the production of smaller conidia.

Type: **China**: *Shandong province*: Kunyushan National Forest Park, Yantai city, from the gallery of *Cryphalus piceae* on *Pinus* sp., 2 Sep. 2020, *R. L. Chang* (HMAS249924-holotype; SNM650 = CGMCC3.20247 – ex-holotype culture).

Description: *Sexual morph* unknown. *Asexual state* hyalorhinocladiella-like: the *conidiophores* may directly arising singly from the vegetative hyphae, (2.4–) 4.7–26.7 (–46.4) µm × (0.8–) 1.0–1.5 (–1.8) µm (Type 1, Fig. 4d, e); or a short basal cell which continues to develop short lateral and terminal extensions with condiogenous sites at their apices or discrete basal cells that produce 1–5 branches, which then branch irregularly and form conidiogenous cells at their apices, (12.2–) 6.2–10.2 (–50.7) µm long (Type 2, Fig. 4b, c); *conidiogenous cells* (4.7–) 6.2–10.2 (–12.4) × (0.7–) 0.9–1.3(–1.5) µm (Fig. 4b, c); *conidia* hyaline, smooth, unicellular short oblong, with rounded ends, (1.1–) 1.4–2.2 (–2.7) × (0.8–) 0.9–1.2 (–1.5) µm (Fig. 4b-e).

Culture characteristics: *Colonies* light brown on MEA (Fig. 4a). *Mycelia* white, superficially growing on the agar. The optimal temperature for growth was 30–35°C, reaching 43.0 mm diam in 10 days. No growth observed at 5°C.

Distribution

Currently known from Yantai City in Shandong Province, China.

Note

Ceratocystiopsis yantaiensis is phylogenetically close to *C. manitobensis* but formed a distinct clade on both ITS and BT trees (Fig. 1). Two types of hyalorhinocladiella-like asexual state were also observed in *C. manitobensis* (Hausner et al., 2003). Conidia of *C.*

yantanensis and *C. manitobensis* are similar in morphology, but the former is smaller in size (Fig. 4b-e).

Taxon 2

Ceratocystiopsis weihaiensis R.L. Chang & X.Y. Zhang, sp. nov.

— MycoBank MB839253; Fig. 5

Etymology

Name refers to Weihai City, where this fungus was isolated.

Diagnosis

Compared to other closely related species, *C. weihaiensis* produces smaller conidia.

Type: **China**: *Shandong province*: Zhujiajuan village, Huancui District, Weihai City, from the gallery of *Cryphalus piceae* on *Pinus* sp., 2 Sep. 2020, *R. L. Chang* (HMAS 249923-holotype; SNM649 = CGMCC3.20246 – ex-holotype culture).

Description: *Sexual morph* unknown. *Asexual state* hyalorhinocladiella-like: the *conidiophores* directly arise singly from the vegetative hyphae, (2.6–) 10.9–29.2 (–44.6) µm × (0.7–) 0.9–1.3 (–1.6) µm (Fig. 5b-e); *conidia* hyaline, smooth, unicellular short oblong, with rounded ends or clavate, ellipsoidal to ovoid (1.5–) 2.0–2.6 (–2.9) × (0.7–) 0.9–1.2 (–1.5) µm (Fig. 5b-e).

Culture characteristics: *Colonies* light brown on MEA (Fig. 5a). *Mycelia* white, submerged in the agar. The optimal temperature for growth is 30°C, reaching 46.0 mm diam in 10 days. Growth is slower at 35°C, 27 mm diam in 10 days.

Distribution

Currently known from Weihai City in Shandong Province, China.

Note

Ceratocystiopsis weihaiensis is phylogenetically close to *C. minuta* but formed a distinct monophyletic clade on both ITS and BT trees (Fig. 1). In the phylogenetic study of *C. minuta* by Plattner et al. (2009) using ITS, LSU and BT gene regions, the authors suggested that this taxon is possibly an assemblage of multiple species. Therefore, they suggested strain RJ705 as the neotype. Later, strain RJ705 = UAMH 11218 = WIN(M) 1532 was designated as the lectotype for *C. minuta* (Reid & Hausner, 2010). The Hyalorhinocladiella-like asexual state was observed in *C. minuta* and closely related species (Plattner et al., 2009). Conidia of *C. weihaiensis* and *C. minuta* are similar in shapes but differs in dimensions (Fig. 5b-e). Conidia of *C. weihaiensis* is smaller than *C. minuta* (2–4 µm × 1–2 µm)(Reid & Hausner, 2010).

Taxon 3

Graphilbum translucens R.L. Chang & X.Y. Zhang, sp. nov.

— MycoBank MB 839254; Fig. 6

Etymology

The name refers to the translucent appearance of the colony on MEA.

Diagnosis: *Graphilbum translucens* differs from closely related species *Gr. puerense* and *Gr. acuminatum* by the shorter hyalorhinocladiella-like conidiophores, smaller conidia and missing pesotum-like asexual state.

Type: **China**: *Shandong province*: Zhujiajuan village, Huancui District, Weihai City, from the gallery of *Cryphalus piceae* on *Pinus* sp., 10 Oct. 2020, *R. L. Chang* (HMAS 249925-holotype; SNM144 = CGMCC 3.20263 – ex-holotype culture).

Description: *Sexual morph* unknown. *Asexual state* hyalorhinocladiella-like: the *conidiophores* directly arising from the vegetative hyphae, (3.6–) 8.6–42.2 (–72.3) µm × (0.9–) 1.1–1.7 (–2.0) µm (Fig. 6b-e); *conidia* hyaline, smooth, unicellular short oblong, with rounded ends or ellipsoidal to ovoid (2.1–) 2.4–3.5 (–4.1) × (0.8–) 1.3–2.0 (–2.7) µm (Fig. 6b-e).

Culture characteristics: *Colonies* light brown on MEA (Fig. 6a). *Mycelia* submerged in the agar. The optimal temperature for growth is 30°C, reaching 74.0 mm diam in 5 days. Growth slower at 35°C, 24 mm diam in 5 days. No growth was observed at 5°C.

Distribution

Currently known from Qingdao City and Weihai City in Shandong Province, China.

Note

Graphilbum translucens is phylogenetically close to *Gr. puerense* and *Gr. acuminatum*. In the ITS tree, *Gr. translucens* grouped with *Gr. puerense* and *Gr. acuminatum* whereas formed distinct clades in BT and EF trees (Fig. 2). The Hyalorhinocladiella-like asexual state was observed in *Gr. translucens* and *Gr. puerense*, but absent in *Gr. acuminatum* (Chang et al., 2017; Jankowiak et al., 2020). The conidiophores of *Gr. translucens* are shorter than *Gr. puerense* (Chang et al., 2017). Conidia of *Gr. translucens* and *Gr. puerense* form hyalorhinocladiella-like asexual state that are similar in shapes (Fig. 6b-e), yet the conidia size of *Gr. translucens* is smaller than *Gr. puerense* (Chang et al., 2017). Unlike *Gr. puerense* and *Gr. acuminatum*, pesotum-like asexual state was not observed among the isolates of *Gr. translucens.*

Taxon 7

Sporothrix villosa R.L. Chang & X.Y. Zhang, sp. nov.

— MycoBank MB 839255; Fig. 7

Etymology

The name refers to the velvety colony morphology of this fungus on MEA.

Diagnosis: *Sporothrix villosa* differ from *S. abietina* by the production of smaller conidia and slow growth rate on MEA at 35°C.

Type: **China**: *Shandong province*: Zhujiajuan village, Huancui District, Weihai City, from *Cryphalus piceae* on *Pinus* sp., 10 Oct. 2020, *R. L. Chang* (HMAS 249926-holotype; SNM188 = CGMCC 3.20264– ex-holotype culture).

Description: *Sexual morph* unknown. *Asexual state* sporothrix-like: the *conidiophores* directly arising from the vegetative hyphae, (3.2–) 6.8–23.8 (–53.6) µm × (0.5–) 0.8–1.3 (–1.5) µm (Fig. 7b, d-e); *conidia* hyaline, smooth, unicellular oblong to ovoid, with rounded ends (1.2–) 1.8–2.6 (–4.1) × (0.7–) 0.8–1.1 (–1.4) µm (Fig. 7c).

Culture characteristics: *Colonies* white on MEA. *Mycelia* submerged in the agar. The optimal temperature for growth is 25°C, reaching 21.1 mm diam in 10 d. Growth is extremely slow at 35°C 3 mm diam in 10 days. No growth observed at 5°C.

Distribution

Currently known from Weihai City in Shandong Province, China.

Note

Sporothrix villosa is closely related to two isolates recovered from China identified as *S. cf. abietina*. This taxon is phylogenetically distinct from all other species in the *S. gossypina* complex (Fig. 3). Six et al. (2011) classified all the isolates from China, Canada, the USA, New Zealand, Korea and South Africa that were close to the ex-type cultures on BT tree as *S. abietina.* But these selected isolates did not form a monophyletic clade. Later, in the phylogenies using BT and CAL gene-regions, Chinese isolates of *S. abietina* did not cluster with the ex-type isolates of *S. abietina*. Therefore, these isolates were provisionally identified as *S. cf. abietina* (Romón et al., 2014a; Romón et al., 2014b). Outcomes from our phylogenetic analyses indicated that isolates classified as *S. abietina* (Six et al., 2011) plausibly included several phylogenetic distinct species. *Sporothrix villosa* produces a *Sporothrix*-like asexual morph similar to

other species in the complex. The conidia of *S. villosa* (Fig. 7c) are smaller than *S. abietina* (Marmolejo & Butin, 1990). Unlike *S. abietina*, *S. villosa* can grow slowly at 35°C.

Discussions

In the present study, we collected *Cryphalus piceae* and their galleries from various pine plantations located near Qingdao, Weihai and Yantai cities, Shandong province of China. From these beetles and galleries, we recovered 176 isolates of ophiostomatoid fungi representing five well-defined genera. These genera were *Ceratocystiopsis*, *Graphilbum*, *Graphium*, *Ophiostoma* and *Sporothrix*. Analyses of molecular and morphological data indicated four of the ophiostomatoid fungal species recovered in this study were previously undescribed. Hereby, we described these species as *C. yantaiensis*, *C. weihaiensis, Gr. translucens* and *S. villosa*.

Among the seven ophiostomatoid species identified in this study, resolving the taxonomy for two isolates of *Gr. crescericum* was particularly challenging. Sequences for ITS, EF and CAL from the European isolates of this fungus are available, however, BT is missing (Jankowiak et al., 2020). Whereas in the present study, we successfully amplified the BT gene (along with ITS and EF), but even after repeated attempts we could not amplify the CAL gene. While comparing the EF gene region, our isolates of *Gr. crescericum* and those from Europe had at least seven base pairs difference. Similar interspecific variation for the ITS, EF, BT and CAL genes were also reported from *O. quercus* and *O. tsotsi* (Chang et al., 2017; Taerum et al., 2018). Therefore, in the future, different sets of primers should be designed for amplifying BT and CAL gene regions for *Graphilbum*. This will allow us to demystify the taxonomy of this genus.

Ophiostoma ips was one of the most frequently isolated ophiostomatoid fungi in China and this study (Lu et al., 2009; Chang et al., 2017; Wang et al., 2018; Chang et al., 2019). Across China, this fungus was also found associated with various species of mites and bark beetles (Chang et al., 2017). As reported for *Sporothrix* sp.1, in the symbiotic relation between *M. alternatus*-*B. xylophilus*-ophiostomatoid fungi, *O. ips* substantially influences the survival and reproduction of the other two partners (Niu et al., 2012; Zhao et al., 2013). Earlier, *O. ips* was also isolated from *M. alternatus*, but its specific function in this symbiotic relationship remained unresolved (Zhao et al., 2018). Therefore, it is not unreasonable to hypothesize that this symbiotic fungus also influences the life history and population of its vector and associated nematode.

Cryphalus piceae vectors diverse groups of fungi and nematodes. At least sixty fungal species were found associated with this beetle. Globally, the diversity of fungi that associate with *Cr. piceae* varies greatly (Ohtaka et al., 2002a; Ohtaka et al., 2002b; Jankowiak & Kolarik, 2010; Jankowiak & Bilanski, 2018). In Poland and Japan, the most frequently isolated ophiostomatoid fungi from *Cr. piceae* was *O. piceae*, and *Leptographium europhioides* and *O. subalpinum*, respectively (Ohtaka et al., 2002b; Yamaoka et al., 2004; Jankowiak & Kolarik, 2010). Whereas, in our study, the dominant fungal species was *O. ips*. A similar trend was also reported from other ophiostomatoid fungi-bark beetle relationships such as those with *Ips typographus* and *Dendroctonus valens* (Taerum et al., 2013; Chang et al., 2019). This suggests that the relationship between bark beetles and their fungal associates is casual.

This shift in the diversity of ophiostomatoid fungi that associate with bark beetles is possibly influenced by both climatic factors and host tree species. Previously, Linnakoski et al., (2016b) indicated that temperature can significantly influence the diversity of fungi that associate with bark beetles. This is not an unreasonable hypothesis because the climatic condition in China, Japan and Poland are markedly different so the bark beetle associated with fungal diversity. In China, we isolated these ophiostomatoid fungi from *Cr. piceae* infecting pine trees whereas in Japan and Poland the host was various species of *Abies* (Ohtaka et al., 2002a; Ohtaka et al., 2002b; Yamaoka et al., 2004; Jankowiak & Kolarik, 2010). Besides climate, this difference in the host tree species could have also influenced the diversity of symbiotic fungi associated with *Cr. piceae.* Pitfalls associated with morphological and molecular characterization of ophiostomatoid fungi can also promote taxonomic misidentification, influencing the diversity.

Ophiostomatoid fungi are an enigmatic taxonomic group (De Beer et al., 2013). As reported previously and in the present study, the morphological differences between the species are often slim (De Beer & Wingfield, 2013; Chang et al., 2019). Additionally, marker genes used for phylogenetic identification frequently vary between species complexes (Linnakoski et al., 2016a; Yin et al., 2019). Isolates of ophiostomatoid fungi recovered from *Cr. piceae* in Japan were exclusively identified using morphological characters (Ohtaka et al., 2002a; Ohtaka et al., 2002b; Yamaoka et al., 2004). Whereas those from Poland were predominantly based on ITS sequences (Jankowiak & Kolarik, 2010). Therefore, chances of misidentification are high, which may have influenced the reported diversity of ophiostomatoid fungi associated with *Cr. piceae* from these regions.

In the last decade, more than a hundred ophiostomatoid fungi were reported from China. Among these, almost half were previously undescribed species (Yin et al., 2016; Chang et al., 2017; Wang et al., 2018; Chang et al., 2019; Chang et al., 2020; Wang et al., 2020). Owing to climate change, economic damages caused by these bark beetles and nematodes has exponentially increased in China (Li, 2013; Tang et al., 2021). This initiated studies focusing on the biology and controlling of these beetles (Sun et al., 2013). These studies simultaneously catalogued the diversity of symbiotic fungi associated with these beetles, influencing fungal species discovery (Sun et al., 2013; Zhao & Sun, 2017).

In this study, we recovered seven species of ophiostomatoid fungi including four previously undescribed species from the Shandong province of China. The previous study from this province, reported two new ophiostomatoid fungi associated with *B. xylophilus* and *M. alternatus* collected from two pine species (Wang et al., 2018). Thus far, more than 10 bark beetle species have been reported from this province (Bai, 1985; Zhu et al., 1991). Previous to this study, no attempts were made to isolate ophiostomatoid fungi from Shandong. Therefore, in future, follow-up surveys and isolations from other bark beetle species from the province will likely allow the discovery of several novel ophiostomatoid fungi.

Abbreviations

ITS: internal transcribed spacer; BT: β-tubulin; EF: elongation factor 1-α; CAL: calmodulin; MEA: malt extract agar; ML: Maximum likelihood; MP: maximum parsimony; BI: Bayesian inference

Declarations

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Adherence to national and international regulations

Not applicable.

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Authors’ contributions

Runlei Chang and Meixue Dai designed the research. Runlei Chang, Tengteng Liu, Xiaowen Yuan and Meixue Dai collected the sample. Runlei Chang, Xiuyu Zhang, Hongsi si and Guoyan Zhao isolated the fungi. Xiuyu Zhang performed experiments in the laboratory, analyzed data. Tengteng Liu identified the beetle. Runlei Chang wrote the first draft of the manuscript. Tanay Bose revised the text, taxonomies and phylogenies. All authors read and approved the final manuscript.

Availability of data and materials

All sequences generated for this study (Tables 1) can be accessed via GenBank: https://www.ncbi.nlm.nih.gov/genbank/. Alignments analysed in this study are available at TreeBase: https://www.treebase.org/.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

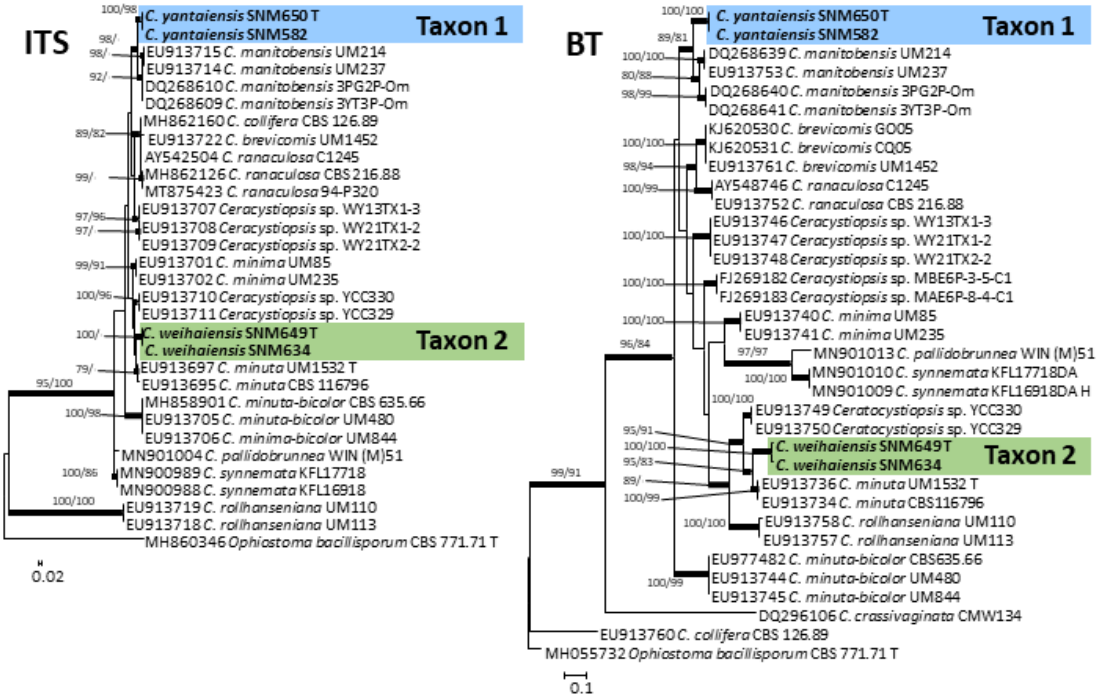

Figure 1

Maximum likelihood phylogeny of Ceratocystiopsis using complete ITS and partial BT gene regions. Isolates recovered in this study are in bold font. ML and MP bootstrap support values ≥ 75 are indicated at the nodes. Bold branches indicate posterior probabilities values ≥ 0.9. T indicates ex-type cultures.

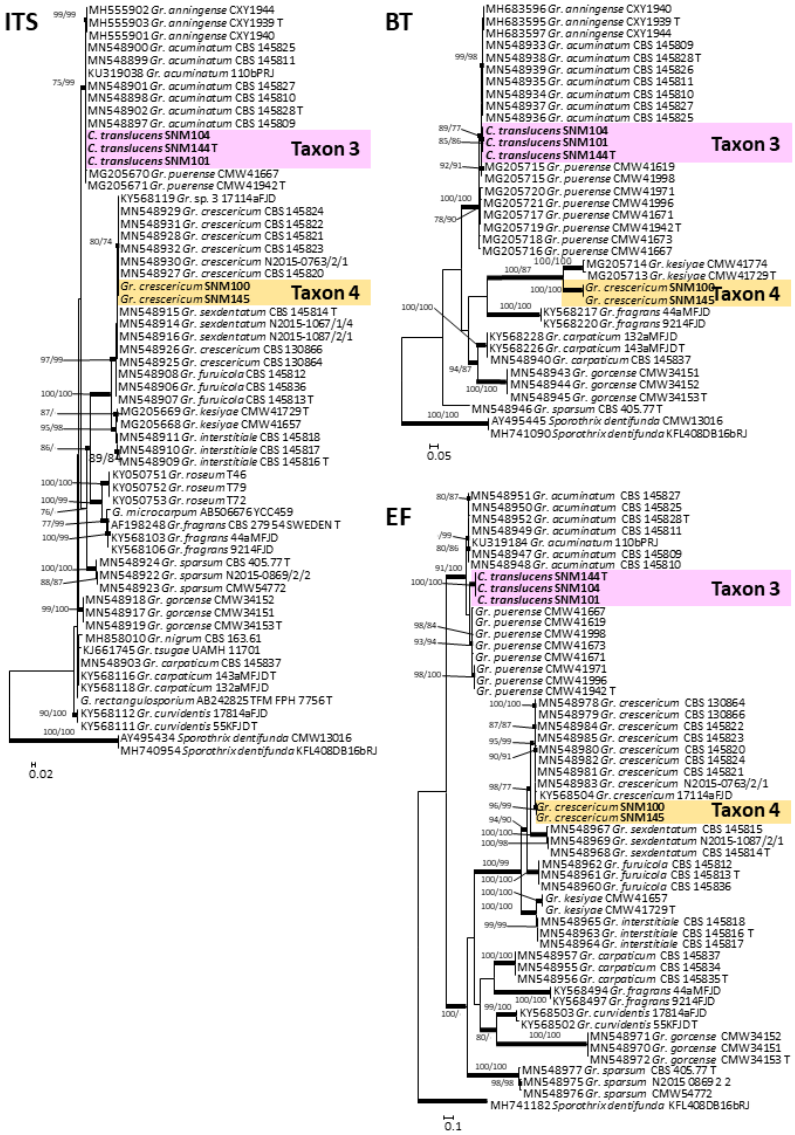

Figure 2

Maximum likelihood phylogeny of Graphilbum using complete ITS, partial BT and partial EF gene regions. Isolates recovered in this study are in bold font. ML and MP bootstrap support values ≥ 75 are indicated at the nodes. Bold branches indicate posterior probabilities values ≥ 0.9. T indicates ex-type cultures.

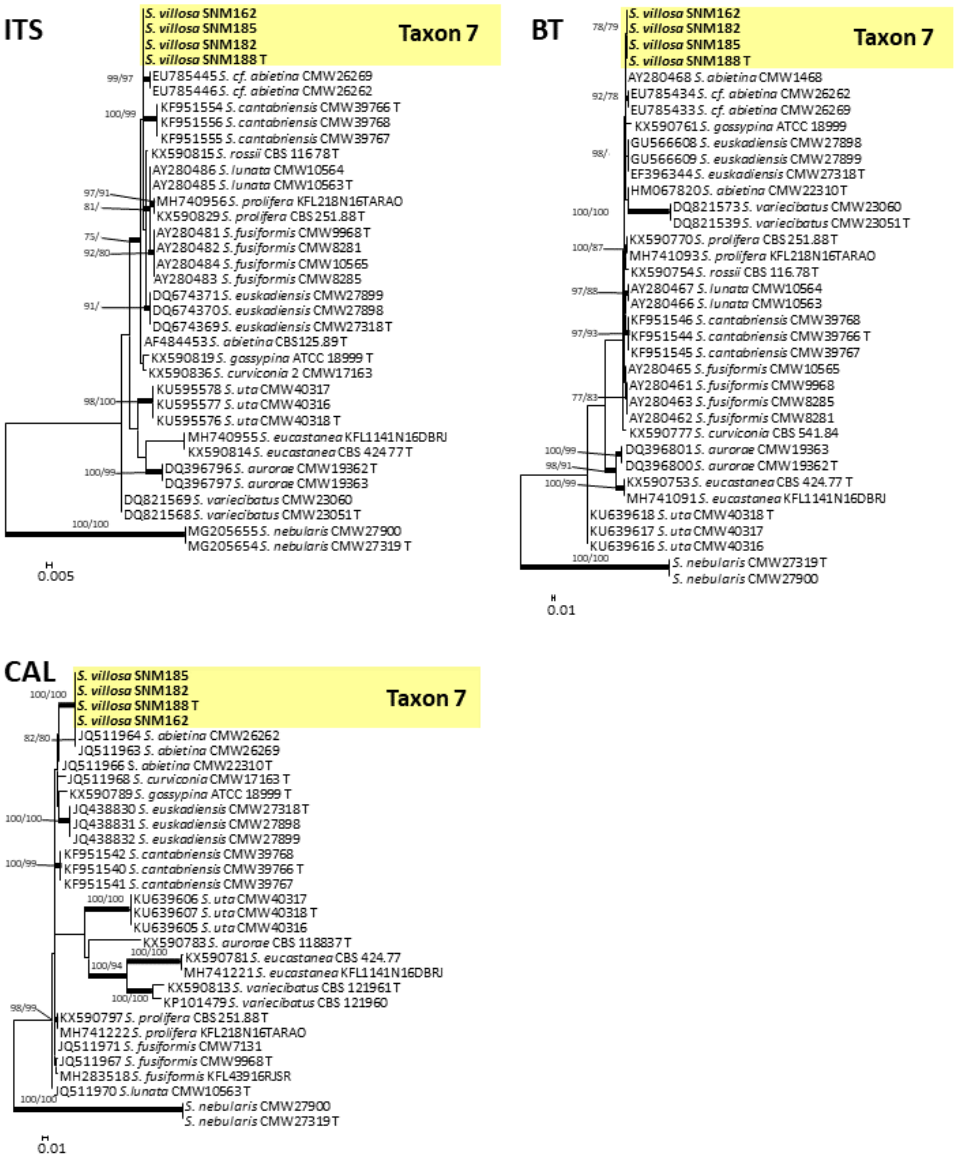

Figure 3

Maximum likelihood phylogeny of Sporothrix gossypina complex using complete ITS and partial BT gene regions. Isolates recovered in this study are in bold font. ML and MP bootstrap support values ≥ 75 are indicated at the nodes. Bold branches indicate posterior probabilities values ≥ 0.9. T indicates ex-type cultures.

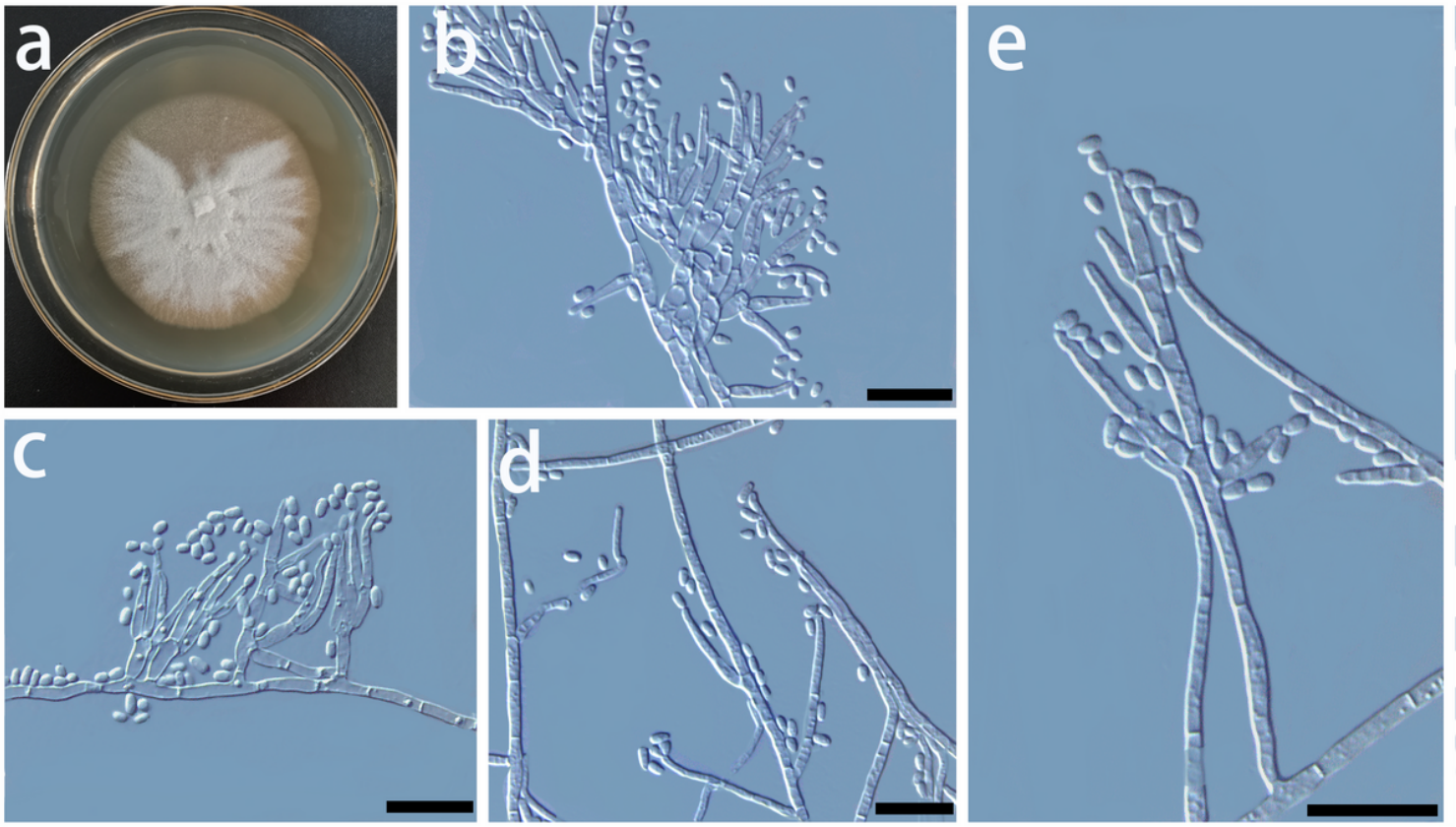

Figure 4

Morphological characters of asexual structures of Ceratocystiopsis yantaiensis sp. nov. (Taxon 1). a. Fourteen-d-old cultures on MEA; b-c. Type 2 hyalorhinocladiella -like asexual morph and conidia; d-e. Type 1 hyalorhinocladiella -like asexual morph and conidia. — Scale bars: 10 μm.

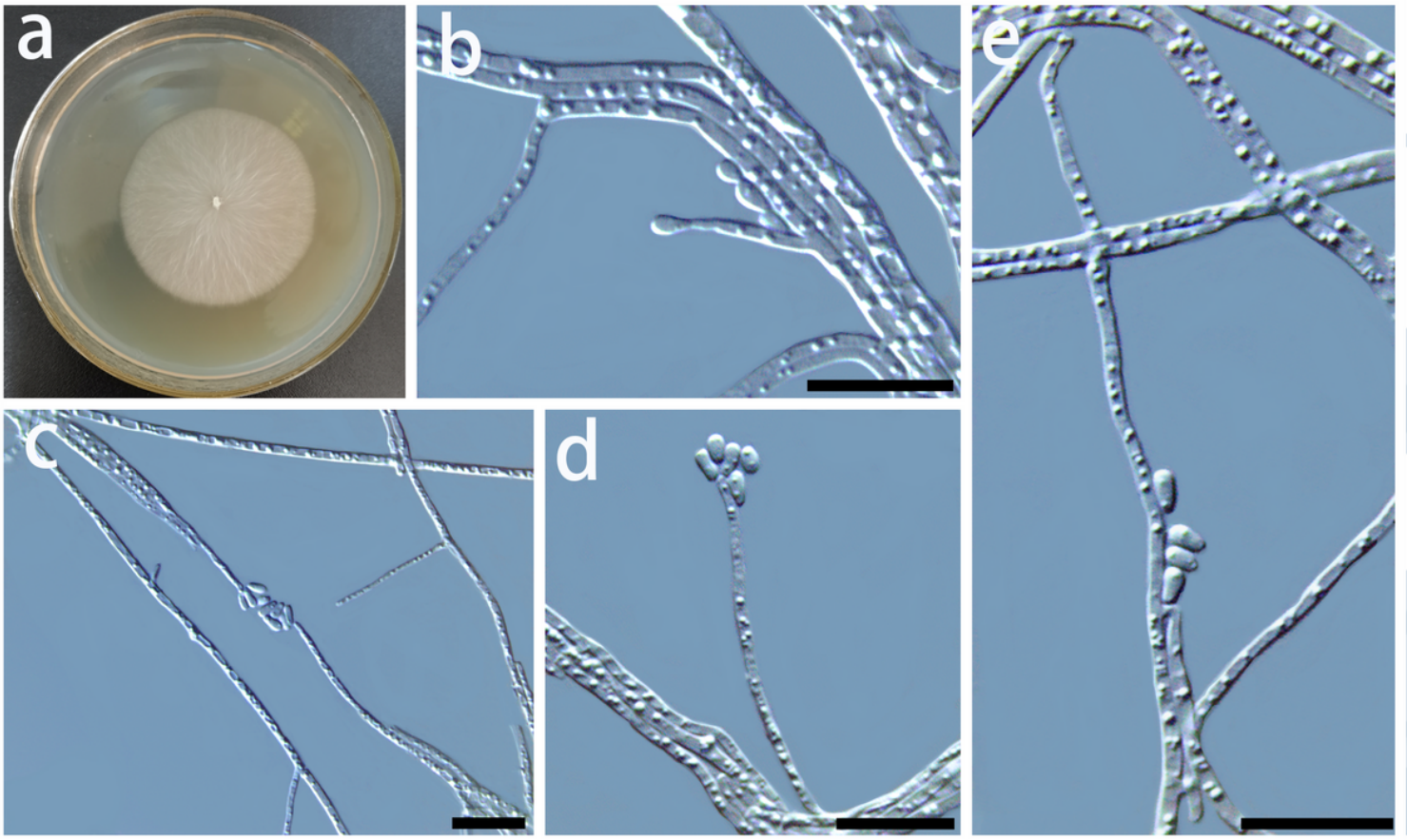

Figure 5

Morphological characters of asexual structures of Ceratocystiopsis weihaiensis sp. nov. (Taxon 2). a. Fourteen-d-old cultures on MEA; b-e. hyalorhinocladiella -like asexual morph and conidia. — Scale bars: 10 μm.

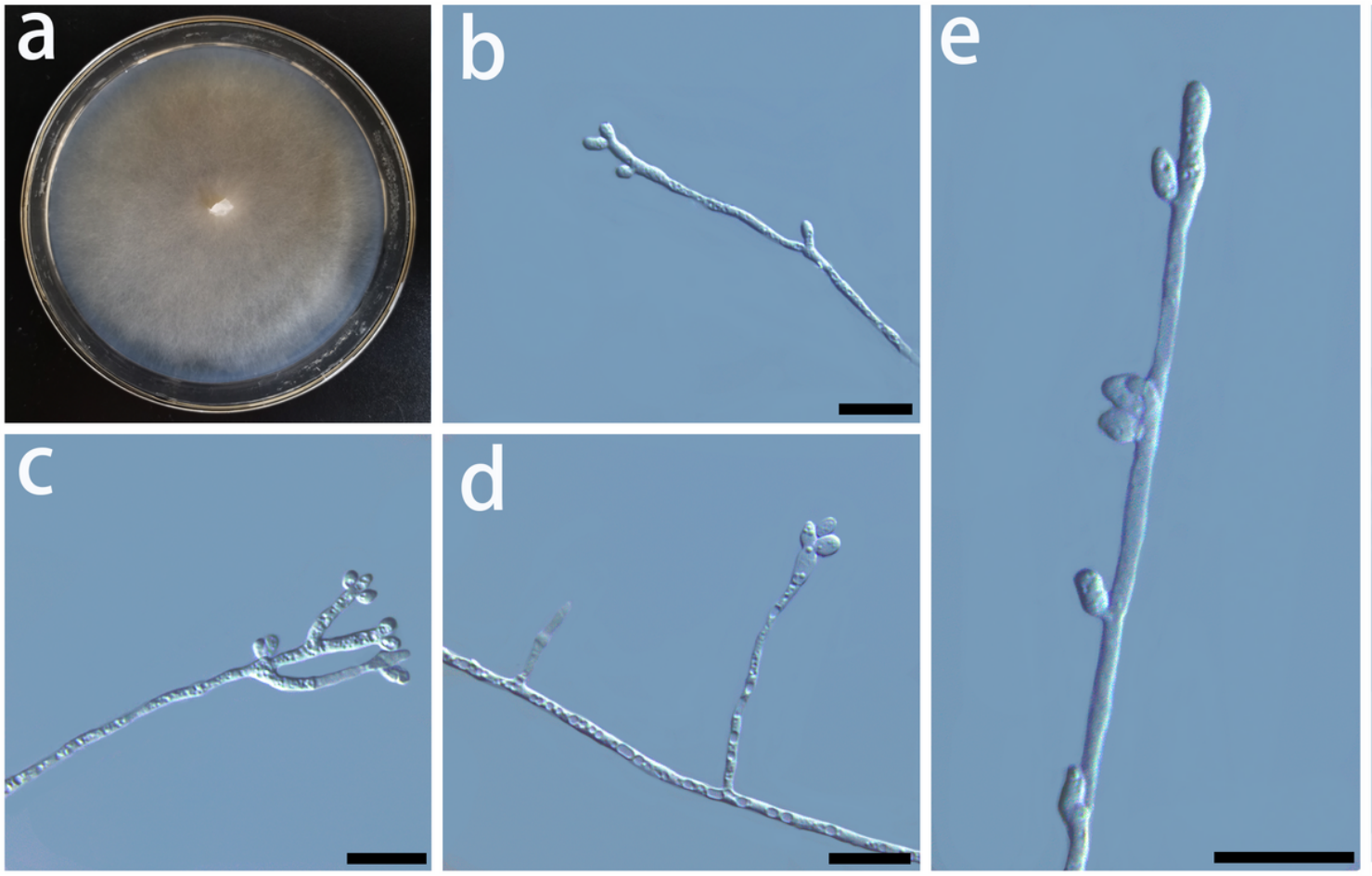

Figure 6

Morphological characters of asexual structures of Graphilbum translucens sp. nov. (Taxon 3). a. Fourteen-d-old cultures on MEA; b-e. hyalorhinocladiella -like asexual morph and conidia. — Scale bars: 10 μm.

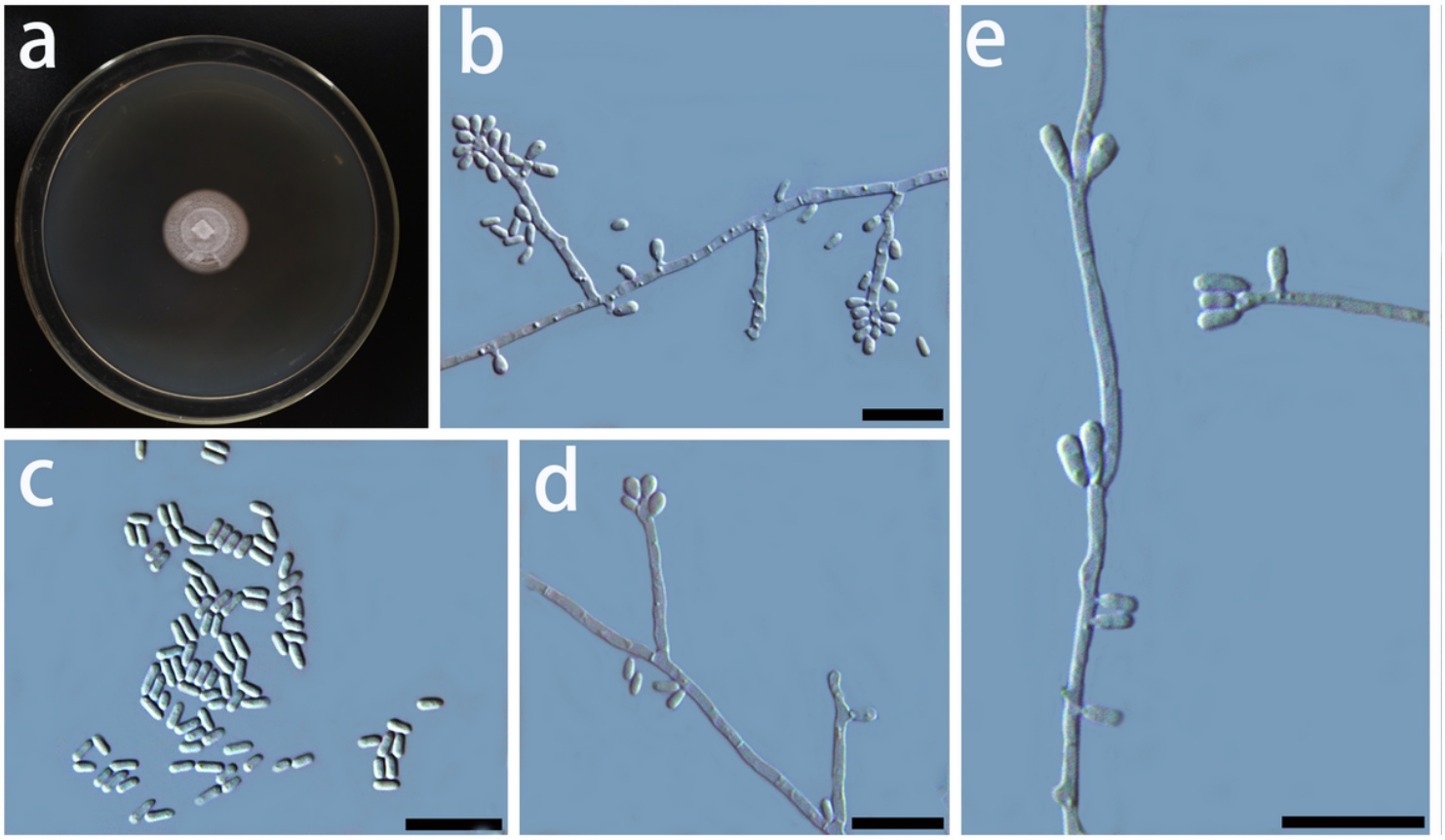

Figure 7

Morphological characters of asexual structures of Sporothrix villosa sp. nov. (Taxon 7). a. Fourteen-d-old cultures on MEA; b-e. sporothrix -like asexual morph and conidia. — Scale bars: 10 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S14.pptx
- Fig.S24.pptx