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DISEASE NOTE

First report of pileus rot disease on cultivated *Morchella importuna* caused by *Diploöspora longispora* in China

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Abstract

In March 2013, pileus rot disease was first observed on cultivated *Morchella importuna*. Infected ascomata were covered by white, velvety mycelia mainly on the pileus, and the infection resulted in malformed fruiting bodies. The causal pathogen was identified as *Diploöspora longispora* based on its morphology and the internal transcribed spacer of its ribosomal DNA sequences. After inoculation of young ascomata with the isolates, the original symptoms were reproduced, and the same pathogen was reisolated from diseased ascomata. This is the first report of pileus rot disease on morels caused by *D. longispora*in China.

Keywords *Diploöspora longispora* · *Morchella importuna* · Morel · Pileus rot disease

The edible, medicinal *Morchella* fungi (morels), mainly *M. importuna* and *M. sextelata*, have been commercially cultivated in fields in China since 2012 (Du et al. 2014; He et al. 2015). In the 2015–2016 production season, the total cultivation area exceeded 1200 ha in more than 15 provinces, municipalities and autonomous regions of China. However, along with the rapid development of morel cultivation, diseases have become serious threats to production. Stipe rot disease caused by the *Fusarium incarnatum–F. equiseti* species complex (Guo et al. 2016) and white mold disease by *Paecilomyces penicillatus* (He et al. 2017) were reported to occur on cultivated *M. importuna* in China. In March 2013, pileus rot disease was first observed on cultivated *M. importuna* in our morel production bases in Pengshui, Chongqing Municipality, Wufeng and Wuhan, Hubei Province, China, and is now common in morel production areas. Approximately 5–8% of the ascomata were found to be infected at a late stage of production in some cultivated sites. Infected ascomata were covered by white, velvety mycelia mainly on the pileus, and the initial lesion expanded rapidly at high temperature (above 25 °C) and relative humidity (more than 90%), causing withering or rotting, and resulted in malformed fruiting bodies (Fig. 1a).

Three fungal strains (designated 60319, 60320 and 60321) were isolated from infected tissues of *M. importuna* in our three morel production bases and cultured on potato dextrose agar (PDA; potato 200 g/L, glucose 20 g/L, agar 20 g/L). After a 14-day incubation at 25 °C, the strains had the same morphological characteristics. Colonies were 4–5 cm in diameter, convex, white, with a smooth margin, and aerial mycelia were sparse to moderate (Fig. 1b). Hyphae were hyaline, smooth, septate, branched, and 2–4 µm in diameter. Conidiophores were micronematous to semi-macronematous, hyaline, and indistinguishable from vegetative hyphae. Conidia were ellipsoidal or citriform, 4–5 × 15–18 µm, 1–3-septate (predominantly 1-septate) (Fig. 1c, d). Conidiogenous cells were blastic or thallic, acropetal or intercalary. Light-brown chlamydospores formed from vegetative hyphal cells in terminal or intercalary clusters of 3–10 cells. They were spherical, with a smooth or verrucose surface, 6–11 µm in diameter, and released mostly in clusters or in short or long chains, and sometimes in pairs or individually (Fig. 1e, f). The cultural and morphological characteristics were consistent with those

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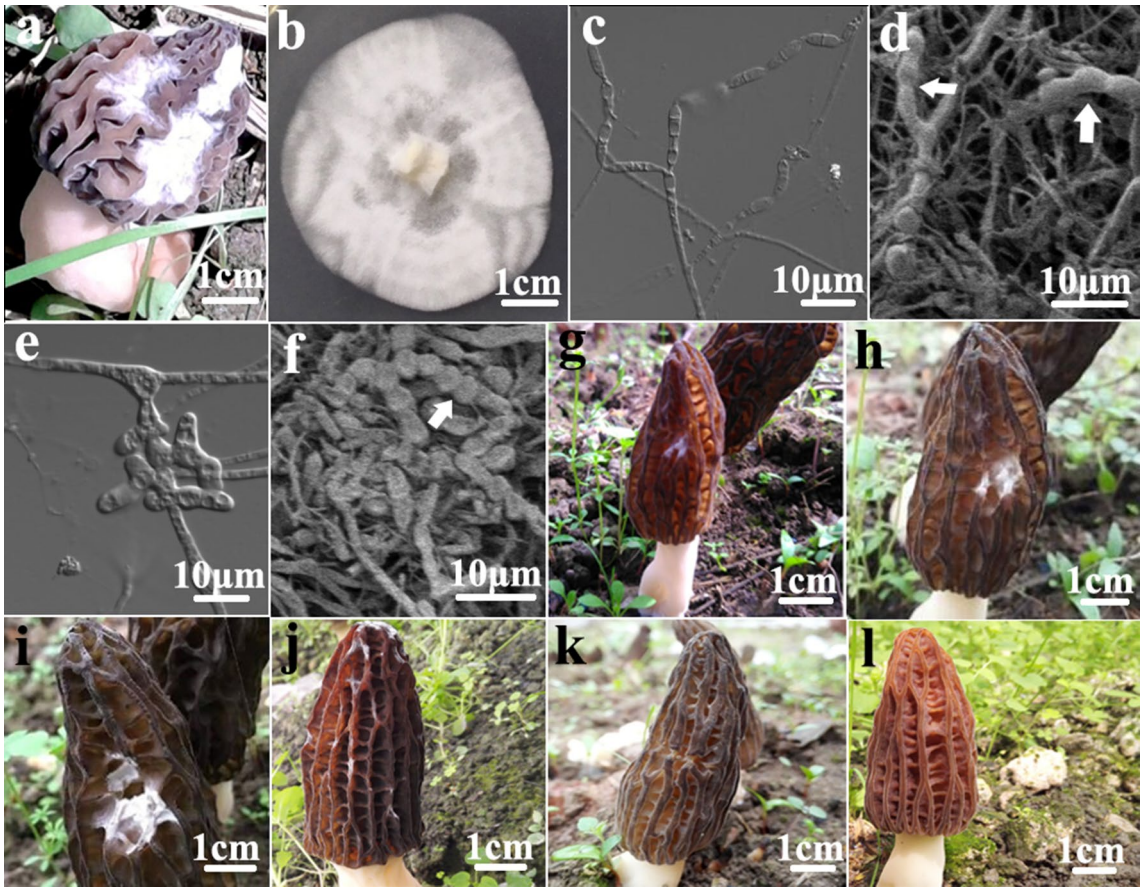

Fig. 1 Pileus rot disease of *Morchella importuna* and its causal fungus, *Diploöspora longispora*. **a** Naturally infected morel. **b** Colony of pathogenic isolate grown on PDA at 25 °C for 14 days. **c** Conidia and conidiophores observed by light microscope. **d** Conidia (arrows) and conidiophores observed by scanning electron microscope (SEM). **e** Chlamydospores and hyphae with chlamydospores in situ observed by light microscope. **f** Chlamydospores (arrow) observed by SEM. Symptoms observed 2, 4 and 6 days after wound-inoculation of healthy morels with fungal suspensions are shown in **g, h** and **i**, respectively. **j** Symptoms observed 6 days after inoculation without wounding. No symptoms developed on the ascomata treated with sterile water after wound-inoculation (**k**) and nonwound-inoculation (**l**)

of *Diploöspora longispora* (Castañeda 1987; Matsushima 1975).

To confirm the identity of the isolated strains, we sequenced and analyzed the ribosomal DNA internal transcribed spacer (rDNA-ITS). Genomic DNA was extracted, rDNA-ITS sequences were amplified using the ITS1 and ITS4 primers (White et al. 1990), and the ITS was sequenced as described previously (He et al. 2015). A partial sequence of 18S rDNA, the complete sequence of ITS1, 5.8S rDNA and ITS2, and a partial sequence of 28S rDNA, were the same among the three strains. A BLAST query of our sequences in GenBank showed that they had more than 99% similarities with *D. longispora* UAMH 340 (GenBank accession KT279806), *D. longispora* UAMH 6367 (GenBank accession KT279807) and *D. longispora* UAMH 6404 (GenBank accession KT279808). Moreover, our sequences also had more than 98% similarities with *Paecilomyces penicillatus* IMI186962 (GenBank accession EU553300) and *P. penicillatus* CBS448.69 (GenBank accession NR_119511). A neighbor-joining phylogenetic tree of the sequence of our strain 60319 (GenBank accession KX223838) and 60320 (GenBank accession KX427537) with related sequences based on the results of the BLAST search was constructed using MEGA5 (Tamura et al. 2011). The results demonstrated that our isolates and reference strains IMI186962 and CBS448.69 of *P. penicillatus* and strain UAMH 340 of *D. longispora* formed a monophyletic clade (Fig. 2), which suggested that similar to *D. longispora* and *D. longispora* var. *cubensis*, our pathogenic cultures were also closely related to several *P. penicillatus* strains, including IMI186962 and CBS 448.69 isolated from mushrooms in Europe (Inglis and Tigano 2006; Luangsa-ard et al. 2004; Tanney et al. 2015). However, apart from conidial chains, there was little morphological similarity between *P. penicillatus* and the pathogenic isolates with acropetal conidiogenesis. Taking into account the morphological characteristics and sequence

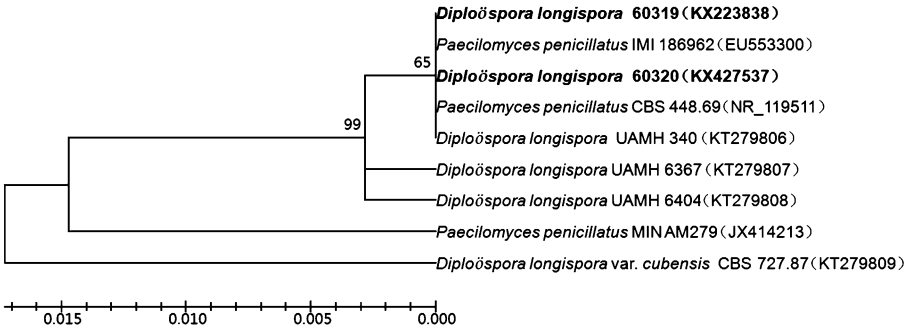

Fig. 2 Neighbor-joining phylogenetic tree based on the ITS sequences of strain 60319, 60320 and related *Paecilomyces* spp. and *Diploöspora* spp. The scale bar indicates genetic distance. Bootstrap values in 1000 replicates for major lineages at the nodes are shown as percentages. Accession numbers are given in parentheses

analysis of rDNA-ITS, the fungus causing pileus rot disease on cultivated *M. importuna* was ultimately identified as *D. longispora*.

Twenty young ascomata of *M. importuna* were inoculated with fungal suspensions of strains 60319, 60320 and 60321, respectively, after wounding or without wounding in different cultivation fields. The pathogen was grown on PDA at 25 °C for 14 days, and the inoculation suspension was prepared by flooding the agar surface with sterilized distilled water to obtain a spore suspension (1×10^5 conidia/mL). For inoculation after wounding, wounds 5 mm long were cut in the pileus of 20 young ascomata with a sterile knife, and 0.2 mL of the spore suspension was applied to the wounds. For inoculation of intact ascomata, 1 mL of the spore suspension was dropped onto the intact young ascomata until run-off.

The results of the inoculations with the three tested strains were consistent. The symptoms mainly occurred on the pileus, but not the stipe of *M. importuna* in both treatments. White, velvety pathogenic mycelia could be seen within 48 h after wounded inoculation (Fig. 1g), and the lesions expanded as the disease progressed, causing withering and rotting (Fig. 1h, i). All 20 wound-inoculated ascomata showed the same symptoms. In the intact treatment, more patches appeared on infected ascomata, and other symptoms were similar to those after the wound inoculation (Fig. 1j). However, compared with the early symptom appearance (within 48 h after inoculation) and 100% infection of ascomata after the wound-inoculation treatment, symptoms on the intact ascomata appeared late (72 h after inoculation), and the percentage of ascomata infected was low (13.3%). The results suggested that *D. longispora* was the biotrophic parasite of cultivated *M. importuna*. In contrast, no symptoms appeared on the ascomata inoculated with sterile water with (Fig. 1k) or without wounding (Fig. 1l). The results implied that colonization by the pathogens may need a long period of high local humidity. Moreover, the same pathogens were reisolated from diseased ascomata and cultured on PDA, satisfying Koch's postulates. The results confirmed that the causal fungus of pileus rot disease on morels was *D. longispora*.

Besides *D. longispora*, our pathogenic strains were also closely related to several *P. penicillatus* strains. The hyphomycete genus *Paecilomyces*is was polyphyletic at the order level. The phylogenetic trees based on sequence analysis of rDNA-ITS suggested that the genus possesses a major division corresponding to the Ascomycete orders Eurotiales and Hypocreales (Inglis and Tigano 2006; Luangsa-Ard et al. 2004), which implied uncertain classification of the genus based on morphological characteristics. The other possible explanation for this phenomenon is an intragenomic variation that is of particular concern for the tandemly repeated rRNA genes (Hibbett et al. 2011). Extensive intragenomic heterogeneity in rDNA-ITS has been recognized in a few fungal taxa, such as *Fusarium* (O'Donnell and Cigelnik 1997), *Cantharellus* (Moncalvo et al. 2006) and *Laetiporus* (Linder and Banik 2011), which may lead to higher estimates of infraspecific variability (Schoch et al. 2012). Furthermore, intergeneric transfer of ribosomal genes between *Thanatephorus cucumeris* and *Ceratobasidium oryzae-sativae* implied the frequent occurrence of fungal intergeneric hybridization and horizontal gene transfer events (Xie et al. 2008). In addition, as a genus incertae sedis in Ascomycota, *Diploöspora* has been poorly studied (Kirk et al. 2008). The sequences of conserved genes available for classification and phylogenetic analysis are also limited. More detailed studies and larger sampling of material belonging to *Paecilomyces* and *Diploöspora* based on morphological characteristics (including asexual and sexual reproduction structures), and the analysis of more conserved gene regions, are needed to clarify the taxonomical status and phylogenetic relationships between these taxa.

Diploöspora longispora has also been isolated from healthy cultivated ascomata of *M. importuna* at high frequencies (around 10%; data not shown), which suggests this fungus might be a morel endophyte. Although plant endophytes have been extensively studied, current knowledge on mycoparasitic fungi is very limited. Watson (1955) isolated by tissue-culture methods, the mycoparasite *Calcarisporium arbuscula* from healthy basidiomata of *Russula* and *Lactarius* species, recognizing it as an endophyte because it did not cause any apparent disease on the parasitized fungi. Fungal

endophytes infect living plant tissues without causing symptoms of disease. However, endophytic fungi are generally closely related to plant pathogens, and some endophytes may also represent latent plant pathogens that have an endophytic stage in their life cycle (Gryzenhout et al. 2009; Junker et al. 2012; Sakalidis et al. 2011). The endophytic and pathogenic lifestyles are not stable but rather are dynamic and likely influenced by the genetic makeup of the fungal species, host factors and changing environment (Delaye et al. 2013; Padhi et al. 2016). When their hosts are under stress due to abiotic pressure, such as sudden and severe climate changes, some endophytic fungi may rapidly cause diseases, becoming a significant threat to agriculture, plantations and native forest ecosystems (Slippers and Wingfield 2007).

Morchella fungi fructify at low temperature (6–16 °C) and moderate relative humidity (around 85%) (Du et al. 2014). *D. longispora* might be able to switch from endophytic to pathogenic under favorable conditions, particularly at temperatures above 25 °C and high relative humidity (> 90%), thereby causing the observed malformations on *Morchella* fruitbodies. These conditions may have in turn increased the distribution of *D. longispora* in soil and air, followed by a greater disease incidence in morel production areas and higher frequency of endophytic isolation.

He et al. (2017) identified the causative agent of white mold disease on cultivated *M. importuna* as *P. penicillatus* using ITS sequence analysis (the morphological characteristics were not provided). The phylogenetic analysis based on rDNA-ITS sequences also placed the pathogen as closely related to *D. longispora* complex (He et al. 2017). The pathogen of pileus rot disease in our study might be different from that of white mold disease. Compared with infection by *D. longispora*, infection by *P. penicillatus* was more serious (more than 60–80% during late harvest stage in 2014 and 2015) (He et al. 2017). Instead of the withering and rotting caused by *D. longispora*, white mold caused perforations of the morel lesions (He et al. 2017). Moreover, white mold may occur on both the caps and the stripes of *Morchella* (He et al. 2017), while *D. longispora* mainly causes pileus rot disease.

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