One of the main culprits of Morchella (Morohellaceae: Morchella) pileus rot disease: Pestalotiopsis trachicarpicola

zhen liang (lz2497818016@163.com)
China West Normal University https://orcid.org/0000-0001-9938-7003
linhui li
https://orcid.org/0000-0002-0915-9793

Research Article

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Abstract

We found some diseased fruiting bodies when cultivating Morchella. The infected ascomata were covered by white mycelia mainly on the pileus, and the infection cause the fruiting body to rot and die. A total of 5 fungi were separated from symptomatic tissue. One of them was proved to be pathogenic by Koch's postulates. The pathogen was identified as *Pestalotiopsis trachicarpicola* based on its morphology and Multi-gene (ITS,*β-tubulin* and *tef1*) analysis. This is the first report of pileus rot disease on cultivated Morchella caused by *P. trachicarpicola* in China, which is of positive significance for the quarantine of cultivated soil of Morchella.

Introduction

Morchella is a kind of edible and medicinal mushrooms with high economic and scientific research value (Badalyan SM and Borhani A, 2019, Tietel Z and Masaphy S, 2018). With the increasing scarcity of wild resources and the expansion of artificial cultivation area by years, there are more and more reports on the cultivation, ecology, genetics, physiology about Morchella (Liu Q *et al.*, 2018, Du XH and Yang ZL, 2021, Du XH *et al.*, 2015). However, so far, Morchella has not yet achieved controllable indoor cultivation, and the open cultivation environment made Morchella vulnerable to pathogenic microorganisms (Liu W *et al.*, 2019). And there are few studies about Morchella diseases. To our knowledge, Morchella pathogens reported at present include *Fusarium incarnatum*(Guo MP *et al.*, 2016), *Fusarium proliferatum*(Lu QY *et al.*, 2019) *Diploöspora longispora*(Peixin *et al.*, 2018), *Cladobotryum protrusum*(Lan YF *et al.*, 2019), *Rhizopus stolonifer*(Zhang YC *et al.*, 2020), these might be only a part of Morel pathogenic fungi, and more remain to be discovered.

During the cultivation of *M. sextelata*, we found a very harmful fungal disease, which caused huge economic losses to the farmers. We speculate that the pathogen may come from the soil, in the early development of *M. sextelata*, it attached to the fruiting body cap, as *M. sextelata* grows, its mycelia gradually invade the interior of the ascomata pileus, eventually make whole fruit body rot and die, result in a serious decline in the quality and yield of *M. sextelata*. We isolated a pathogenic fungi from this nidus, through morphological identification and Multi-gene (ITS,*β-tubulin* and *tef1*) analysis, it was identified as *Pestalotiopsis trachicarpicola.*

Materials And Methods

Pathogen isolation

The infected *M. sextelata* was placed in Clean Bench and the surface of fruiting body was washed with sterile water, then cut the diseased plaque and inoculated on potato-dextrose agar (PDA) medium, cultured at 25 ℃ under dark conditions. The marginal mycelia were picked for isolation of pure culture, when the mycelia began to germinate. The purified pathogen strain is stored at 4 ℃ .

Pathogenicity tests

Koch's postulates was used to verify the pathogenicity of pathogen. Firstly, healthy ascomata were selected in the cultivation field of *M. sextelata* and a 5 mm hole was inserted on the cap with sterile toothpick. Meanwhile, conidia were cultured on PDA plate and eluted to prepare suspension. Then, 20μL suspension was inoculated into the hole and sterile water was used as negative control.

After the fruiting body of *M. sextelata* showed symptoms, isolation of pure culture was carried out again from the diseased spot, and if the pure culture was completely consistent with the previous culture, the fungus was identified to be pathogen.

Morphological observation

The pathogen was inoculated on PDA plate and cultured at 25°C in Darkness for 3–15 days to visually observe the color, diameter and characteristics of hyphae. Approximately 25 random conidia were examined for morphological characters (the size, the shape, color type, the number and length of apical and basal appendages) using Optical Microscope. Identification was based on the keys and

descriptions provided by *General Mycology* (Xing LJ *et al.*, 2021) and *FLORA FUNGORUM SINICORUM VOL.38* (Ge QX *et al.*, 2009).

DNA extraction, PCR amplification and nucleotide sequencing

The pathogenic fungi were grown on PDA plate and cultured at 25°C in Darkness for 9 days. The fresh mycelium was scraped from the surface of the culture medium, and the whole genomic DNA of the pathogen was extracted with a fungal DNA extraction Kit(sangon Biotech, shanghai). The internal transcribed spacer regions, interval 5.8 S nrRNA gene (ITS) was amplified using primer pairs ITS1(5'-TCCGTAGGTGAACCTGCGG-3')and ITS4□5'-TCCTCCGCTTATTGATATGC-3'□(White TJ *et al.*, 1990), partial β-tubulin(*tub2*) gene region was amplified with primer pairs BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2B (5'ACCCTCAGTGTAGTGACCCTTGGC-3')(Maharachchikumbura SSN *et al.*, 2012),and part of translational elongation factors(*tef1*) gene was amplified using the primer pairs EF1-526F (5'-GTCGTYGTYATYGGHCAYGT-3') and EF1-1567R(5'-ACHGTRCCRATACCACCRATCTT-3') (Maharachchikumbura SSN et al., 2012).

PCR was performed in a 25μL reaction system containing 10.3μL of double distilled water, 12.5μL 2×Taq MasterMix(sangon Biotech,shanghai),0.6μl of each primer (10μM) and 1μL DNA template. The thermal cycling program was as follows: For ITS an initial denaturing step of 95°C for 3 min, followed by 30 amplification cycles of 95°C for 30 s, 52°C for 45 s and 72°C for 90 s, and a final extension step of 72°C for 10 min. For *β-tubulin* PCR conditions were an initial step of 3 min at 95°C, 30 cycles of 30 s at 95°C,

50 s at 55°C, and 1 min at 72°C, followed by 10 min at 72°C. For *tef1*, an initial step of 3 min at 95°C,30 cycles of 30 s at 95°C, 55 s at 58°C, 90 s at 72°C(Maharachchikumbura SSN et al., 2012). The PCR products were detected by 1% agarose gel electrophoresis and sent to Sangon Biotech for sequencing.

Phylogenetic analysis

The sequencing results were compared and analyzed in NCBI database by Blast. Similar strains were selected for multi gene joint tree construction. Combination of multi-locus dataset of three gene regions was phylogenetic analyzed by using MEGA7 software(Kumar S *et al.*, 2016). The sequence alignments algorithm adopts ClustalW to delete the unaligned sequence manually after the alignment, and then uses the method of Maximum Likelihood (ML) to build the phylogenetic trees. The branch support of ML analysis was evaluated by 1,000 bootstrap replications.

Results

Pathogen isolation and Pathogenicity tests

In this study, five strains of fungi were isolated from the fruiting body of diseased *M. sextelata* and numbered M-1, M-2, M-3, M-4 and M-5 respectively. It was found that the fruiting body of *M. sextelata* inoculated with M-5 appear obvious lesions by Koch's postulates verification (Fig. 1b), and the symptom of the lesions was consistent with that of the naturally diseased ascomata (Fig. 1a), however, there was no symptom in the control group (Fig. 1c), this showed the fungus M-5 really was a Morchella pathogen.

Morphological characteristics of pathogen

The colony on the PDA reached 90 mm in diameter after 9 days at 25℃, aerial mycelium on surface, edge fimbriate, white and dense, the back was yellow white, and the conidioma was black, round and mucinous (Fig. 2a, b and c). Conidia fusiform, straight to slightly curved, consists of 5 cells, 4-septate, 21.0-28.6×6.0-7.6μm(x̄ = 24.8×7.0μm); apical cell hyaline, conic to subcylindrical 2.7–4.5μm long (x̄ = 3.7μm); with 2–4 tubular apical appendages, arising from the apex of the apical cell, 6.0-19.3μm long (x̄ = 10.2μm);the three intermediate cells of conidia, concolorous to versicolorous, brown to olivaceous, melanin deposition, doliiform to cylindrical, constricted at septa, peripheral and diaphragm walls darker than the rest of the cell, together 13.9–17.6μm long (x̄ = 15.9μm); second cell 4.6–5.9μm long(x̄ = 5.4μm); third cell 4.4-6.0μm long(x̄ = 5.1μm); fourth cell 4.1–6.5μm long(x̄ = 5.39μm); basal cell conic to acute, 3.3–7.2μm long (x̄ = 5.2μm), with a basal appendage,2.4–6.9μm long(x̄ = 4.5μm)(Fig. 2d-g). After consulting (Maharachchikumbura SSN et al., 2012, Ge QX et al., 2009)the morphology of the pathogen M-5 was consistent with that of *Pestalotiopsis spp.*.

Phylogenic study

The PCR amplified fragments were sent to the company for sequencing to obtain the partial sequence of ITS of 586bp, the partial sequence of *β-tubulin* gene of 458bp and the partial sequence of *tef1* gene of 965bp. The three sequences were uploaded to GenBank and the accession numbers were OL362082, OL828342 and OL905009, respectively. The sequencing results were compared on the NCBI website, and the results showed that the three sequences of M-5 strain were identical with *Pestalotiopsis trachicarpicola*, homology is as high as 99% – 100%. The phylogenetic tree was constructed by using MEGA7 software, and the information of the tree was shown in Table 1. The pathogen M-5 strain and *Pestalotiopsis trachicarpicola* strain YB1 were clustered in the same branch, and the test value was as high as 100%(Fig. 3).

The pathogen M-5 was identified as *Pestalotiopsis trachicarpicola* by morphological identification and multi-gene analysis.

Discussion

The pathogen of *M. sextelata* is classified in *Pestalotiopsis spp.*. This genus, is characterized by five cells and the middle three by dark color, which is a fungi imperfecti and was established and named by Steyaert in 1949 (Steyaert RL, 1949). It is very difficult to classify and identify (Maharachchikumbura SSN *et al.*, 2011). At present, the morphology of conidia is the most widely used taxonomic feature of *Pestalotiopsis spp.*, which is classified by the length, width, cell color, number and length of attached filaments of conidia(Jeewon R *et al.*, 2004).However, because of the morphological similarity between species, only observing the morphology of conidia is not enough to accurately identify the species. Therefore, the molecular data are also gradually used in the classification of this genus, and the classification and identification system of this genus is gradually improved from single analysis of conserved ITS sequence to joint analysis of multiple genes(Liu AR *et al.*, 2010). Now, the most commonly used multi-gene joint analysis is through the combination of three sequences: ITS sequence, *tub2* and *tef1*, which shows better differences among different species(Maharachchikumbura SSN et al., 2012). In this study, the pathogen of *M. sextelata* was identified as *Pestalotiopsis trachicarpicola* by the method described above.

Pestalotiopsis spp. is an important plant pathogen, which causes diseases all over the world. It can not only cause diseases of seeds and seedlings, but also cause leaf spot, branch and trunk blight and fruit rot. Among them, leaf spot is the most common, and the main host includes *Camellia sinensis*(Maharachchikumbura SSN *et al.*, 2016, Wang YC *et al.*, 2019, Chen YJ *et al.*, 2017), *Photinia × fraseri*(Zhu YH *et al.*, 2021, Xu XL *et al.*, 2021), *Paris polyphylla*(He X *et al.*, 2020),*Vitis davidii*(Tang XB *et al.*, 2020), *Banana*(Bhuiyan MAB *et al.*, 2021), *Strawberry*(Morales-Mora LA *et al.*, 2019), *Eucommia ulmoides*(Li SJ *et al.*, 2018), *Mango*(Shu J *et al.*, 2019)and so on. Its typical disease symptoms on the leaf consisted of circular, semicircular or irregular shape of the disease spot, brown at early stage and grayish brown to grayish white at middle stage and eventually lead to leaf abscission. Branch and trunk blight often occurs on *Blueberry Bushes*(Borrero C *et al.*, 2017), *Chinese Hickory*(Liu YJ *et al.*, 2014), *Loquat*(Fang L *et al.*, 2013), *Pinus bungeana*(Qi M *et al.*, 2021) and grapevine(Jayawardena RS *et al.*,

2015). Initially, the necrotic lesions appeared on the branch and trunk, which led to the discoloration and cracking of the bark with the development of the disease, branch and trunk with the lesion were wilt at the end. Fruit rot mainly occurs in the harvest and storage period of *Grape*(Ghuffar S *et al.*, 2018, Jayawardena RS et al., 2015), *kiwifruit*(Li L *et al.*, 2016), *rambutan*(Keith LM, 2008), *Guava*(Keith LM *et al.*, 2006), *Persimmon* (Palou L *et al.*, 2015)etc. Dark brown to black spots appears in the disease spot at early stage and white hyphae grow in the later stage, whole fruit rots at the end. If the fruit is damaged, it is more likely to be infected. The infection phenomenon of each species will be different. In addition, it also causes the problems of *Cunninghamia lanceolata* (Zeng SH, 1998, Huang TZ, 1983), *strawberry*(Hua XF *et al.*, 2021) rotten seed, rotten root and seedling growth decline. However, so far, the pathogenic effect of this genus on macrofungi has not been found.

It has been reported that the *Pestalotiopsis spp.* mainly take the soil, diseased seedlings and organization as the transmission carriers, which prefer to grow in the high humidity environment. The younger the infected tissue is more likely to get infected and tissue damage is also conducive to pathogen infection (Ge QX et al., 2009). Morchella is suited to grow in high humidity environment, and its tissue is very fragile and vulnerable to mechanical and insect damage, which increase the possibility of Morchella infection (Ruíz-Herrera J and Osorio E, 1974, Sambyal K and Singh RV, 2021). Most importantly, as an edible commercial mushroom, Morchella grows and is harvested in a short time(Zhi CY *et al.*, 2021). Once infected, it loses all commercial value, therefore, we should pay more attention to the soil quarantine work in the cultivation process of Morchella.

Declarations

Informed consent statement

All authors have seen a copy of the manuscript and have approved its submission.

Author contribution

LHL conceived the idea and designed the experiments. ZL carried out the research experiments and analyzed the results. ZL drafted the manuscript. LHL revised the manuscript.

Data Availability Statement

Some or all data, models, or code generated or used during the study are available from the corresponding author by request. (List items).

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

funding

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Table

Table 1 is only available as a download in the Supplemental Files section.

Figures

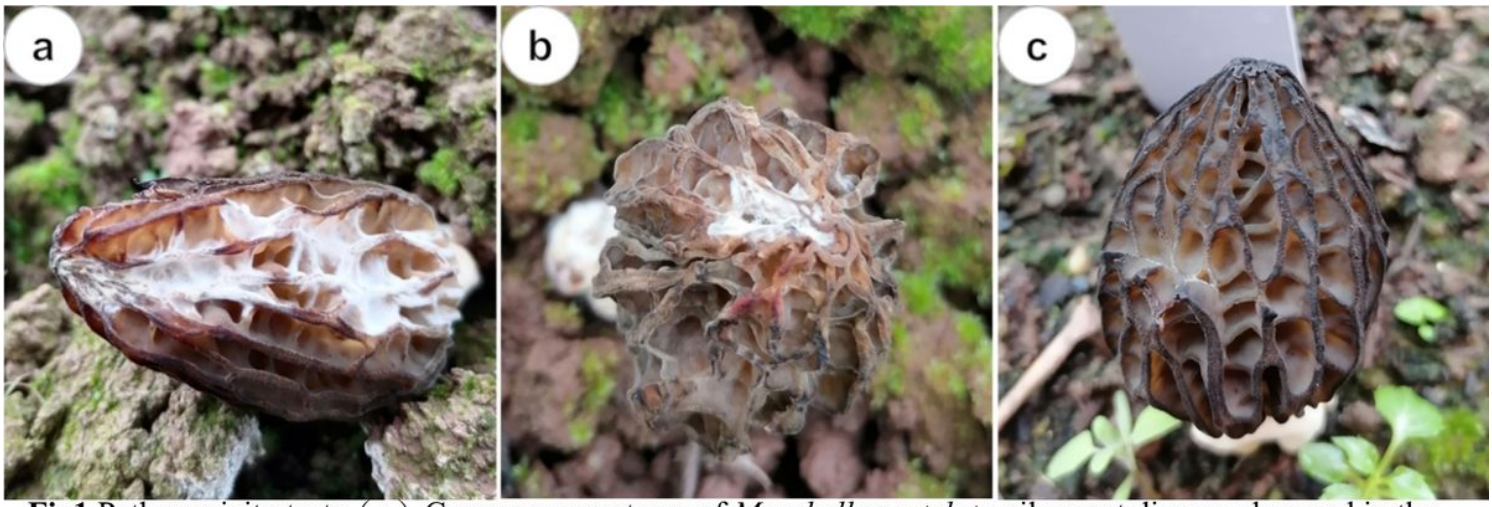

Fig1 Pathogenicity tests（a） Common symptoms of *Morchella sextelata* pileus rot disease observed in the field（b） Pathogenicity tests of M-5 strain On *Morchella sextelata*（c） No disease symptoms were observed on the control *Morchella sextelata*

Figure 1

Please See image above for figure legend.

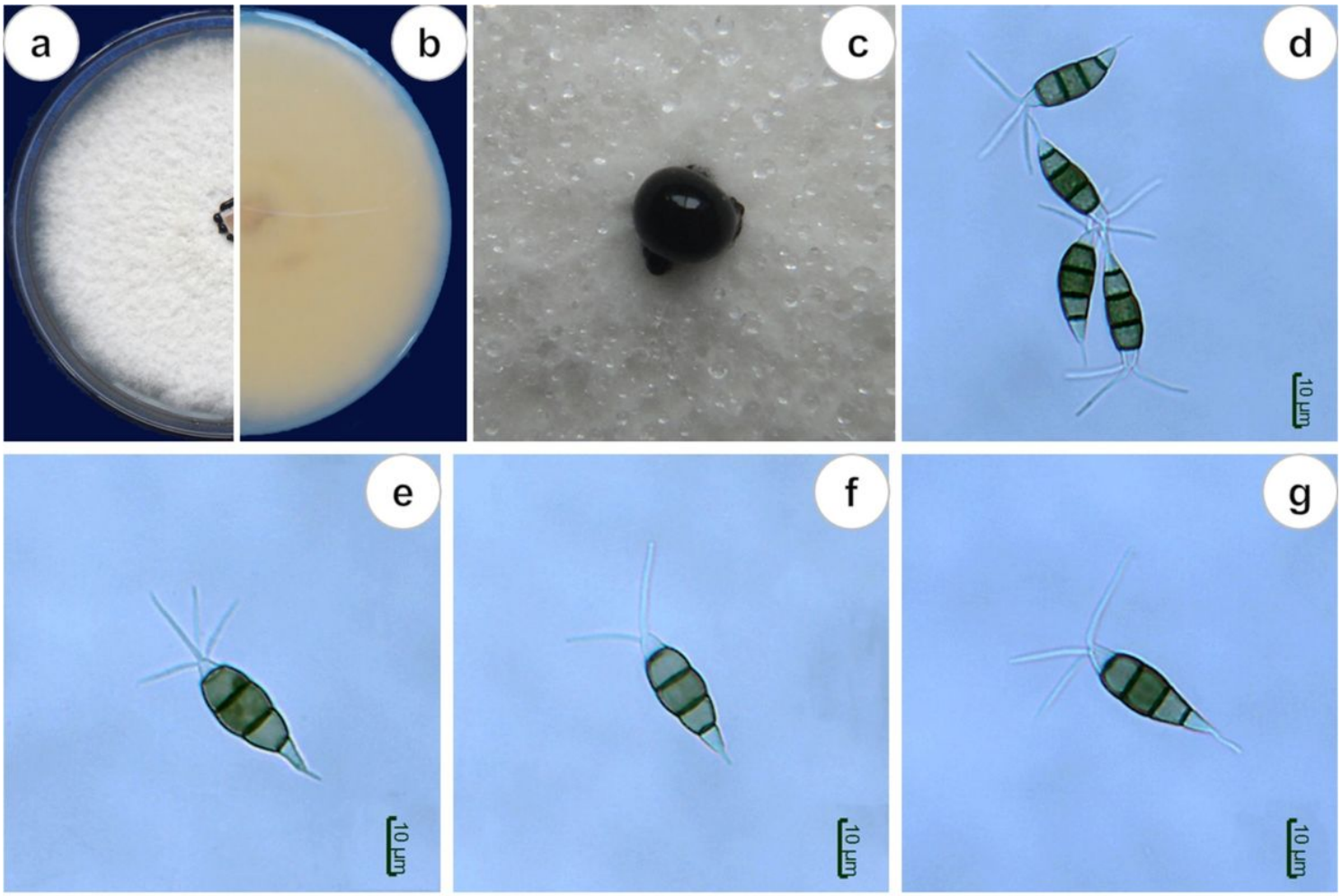

Fig2 Morphological characteristics of pathogen M-5 (a, b) Colony on PDA, (a) from above, (b) from below, (c) Conidiomata, (d-g) Conidia with concolorous and versicolorous median cells

Figure 2

Please See image above for figure legend.

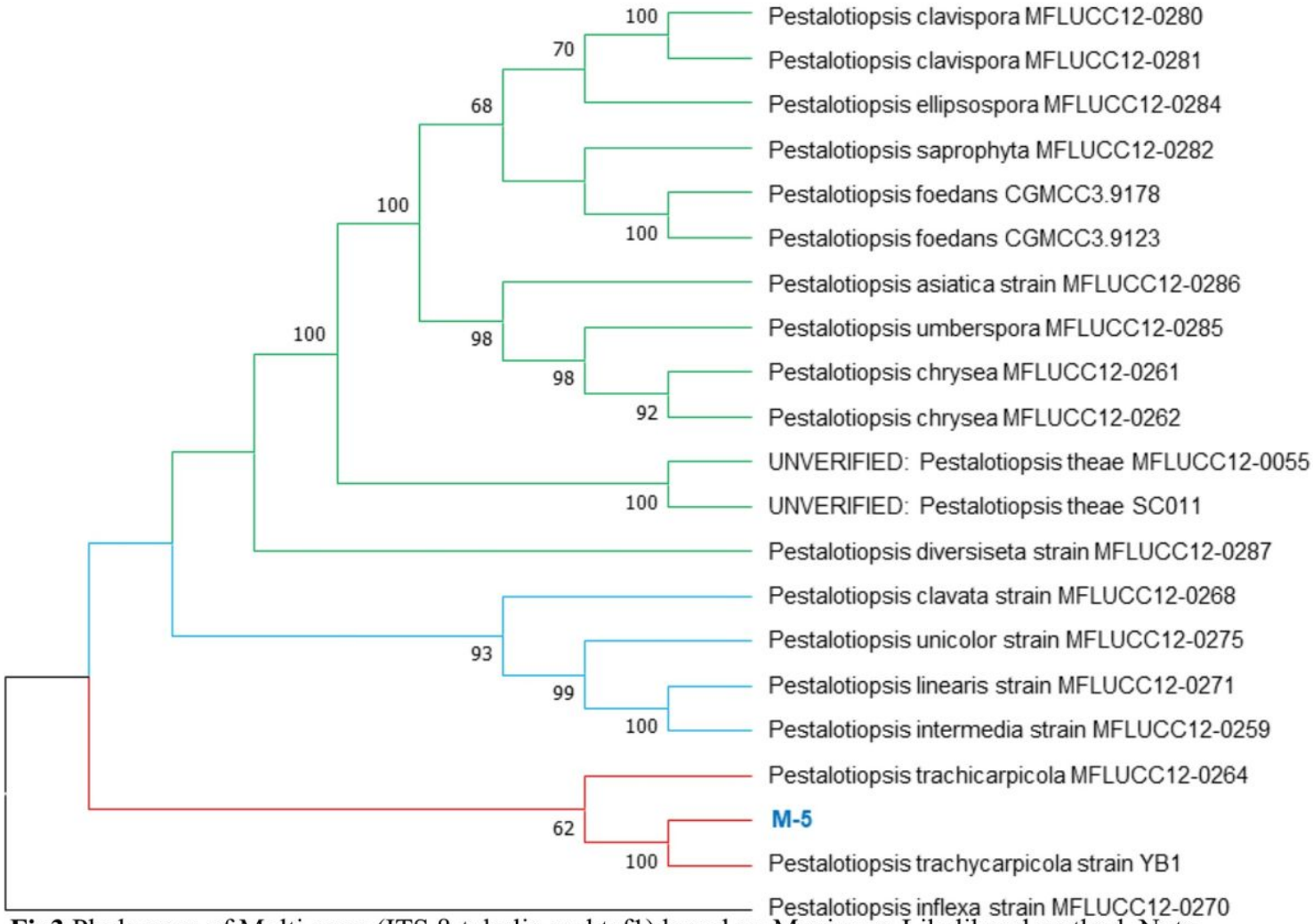

Fig3 Phylogram of Multi-gene (ITS,β-tubulin and tef1) based on Maximum Likelihood method. Note Numbers above the twigs were presented as bootstrap values greater than 50%. The GenBank accession number is in another table, where only the strain number is reflected. The pathogen M-5 is highlighted in red

Figure 3

Please See image above for figure legend.

Supplementary Files

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- Tab1.png