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REVIEW PAPER

# *Pestalotiopsis trachicarpicola*, a novel pathogen causes twig blight of *Pinus bungeana* (Pinaceae: Pinoideae) in China

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**Abstract** *Pinus bungeana* is one of indigenous trees in China and widely distributed in poor and arid regions for vegetation and industrial woody use. However, since a high-incidence disease threatens the growth of mature *P. bungeana* tree in the garden and in the plantation every year, the overwintering shoots were infected and died in the next spring with a ratio over 70%, but the cause was beyond understood. A total of 120 fungal isolates were separated from symptomatic twigs by histological isolation methods, including *Pestalotiopsis* spp., *Fusarium* spp., *Trichothecium* spp., *Penicillium* and some unknown fungal species. *Pestalotiopsis* spp. was dominant, accounting for 85%. Morphological observation under microcopy showed all *Pestalotiopsis* species are identical, and six isolations among them were randomly selected for pathogenicity tests. Fulfilling Koch's postulates showed that all six isolates of *Pestalotiopsis* spp. were pathogens of twig blight, causing the same symptoms as observed in the field, while other non-*Pestalotiopsis* isolates were avirulent to *P. bungeana* twigs. Multi-gene (*ITS, tub2* and *TEF1*) analysis and morphological observation revealed that all the six *Pestalotiopsis* isolates belonged to *P. trachicarpicola*. To our knowledge, this is the first study reporting *P. trachicarpicola* as the pathogens responsible for *P. bungeana* twig blight in China.

**Keywords** *Pestalotiopsis trachicarpicola* · *tub2* gene · *ITS* · *Pinus bungeana* · Morphology · Twig blight

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## Introduction

*Pinus bungeana* Zucc. ex Endl. is an evergreen conifer species within the family Pinaceae and is naturally distributed in Central and Western China, which is across warm temperate, northern subtropic, and mid-subtropic climatic zones, geographically ranging from 29° 55′ to 38° 25′ N latitude and from 103° 36′ to 115° 17′ E longitude. This region covers 7 provinces, three (Shaanxi, Shanxi, and Henan) of which are the main distribution areas of an altitude from 500 to 1800 m (Jie and Kentian 1993; Fu et al. 1999; Wang and Liu 1998; Li et al. 2016; Guo et al. 2020). *P. bungeana* effectively adapts to drought and cold climates as well as calcareous loess and mild saline soil, so it is widely used as afforestation tree species (Bo 2008). It is a popular ornamental plant that has strong resistance to sulfur dioxide and soot pollution in the air. In addition, its cones and pollen are valuable medicinal herbs, the turpentine is an important chemical raw material, and the edible seeds are known as pine nuts (Zhao et al. 1995; Wang et al. 1999). Therefore, *P. bungeana* is an

economically and ecologically important soft conifer species with a key role.

In recent years, due to the over-cutting and fragmentation of natural habitats, the wild resources of *P. bungeana* forests have increasingly declined, but artificial plantation area increased. What follows are the advent of many fungal, bacterial and viral diseases and the consequent problems for *P. bungeana*. In the field, initial symptoms of twig blight disease are mostly found occurring at the new overwintering shoots since Nov. in central China. The current year twigs are attacked by canker spots, then swollen bark and exudates are generated consequently. After the canker spots circle around the whole bark of twigs, the above-canker part of the twig turns to be blighted and dead. The whole process usually lasts 3 months.

There are a few reported diseases on *P. bungeana,* including needles castle caused by *Lophodermium anhuiense*, which is commonly distributed throughout the China and damages the forest heavily under stressed environments (Lin and Tang 1988), trunk rot caused by *Valsa pini* occasionally in Hebei province, northern China (Pan et al. 1998) and biotrophic disease caused early defoliation by *Coleosporium solidaginis* in Japan and China (Kaneko 1981). But no report about twig blight in *P. bungeana* was ever found till now. In this paper, we fulfilled the Koch's postulates for this novel pathogen and limited its taxonomical level by combining morphological and molecular phylogenic descriptions.

## Materials and methods

### Pathogen isolation and Morphological observation

Symptomatic and non-symptomatic twigs of *P. bungeana* were collected from 2 gardens and 4 plantations in Xingping, Shaanxi Province of China in November 2017 and 2018, including a total of 32 trees and 60 samples of twigs. The twigs were washed with running tap water, then sterilized with 75% ethanol (60 s), 1.3% NaClO (2 min) and 75% ethanol (30 s) (Wei and Xu 2004). The samples were washed three times with sterilized water, then cut into pieces of 1 cm long and placed on potato-dextrose agar (PDA) medium at 25 °C chamber for 3–20 days. Pure fungal cultures were obtained by single spore isolations following the methods outlined by Lacap et al. (2003) and Promputtha et al. (2005).

The color of the mycelium on PDA was visually observed after incubated for 7 days. Then the mycelium and the fruiting body were observed for more morphological characters using a light microscope. Approximately 30 random conidia were examined for morphological characters (the size, color type shape, the number and length of apical and basal appendages) using a Leica DM4000B (Leica, Germany). Identification was based on the keys and descriptions provided by Steyaert (1949), Benjamin and Guba (1960) and Sutton (1980).

### Pathogenicity tests

Six isolates were selected randomly from the *Pestaloptisis* spp. colonies for pathogenicity tests. Eight detached 1-year-old twigs were taken from mature *P. bungeana* trees. Branch periderm of 1 cm diameter hole was punched and ready for inoculation. PDA plugs (Diameter 0.5 cm) with mycelia and conidia from a 7-day-old culture were placed in the punched hole. Two controls were treated with blank PDA plugs. The inoculation sites were covered by wet cotton to keep saturated moisture, then the inoculated twigs as well as the controls were put into 90-mm sealed petri plates with wetted filter paper at the bottom. All twigs were maintained in the greenhouse with 10 h light and 14 h dark per day at 25 °C for 7 to 10 days. The other non-*Pestaloptisis* isolates were all implemented for pathogenicity tests as above methods, each on 3 twigs. Symptomatic twigs were cultured and fungal samples were re-isolated and identified.

### DNA extraction, PCR amplification and nucleotide sequencing

The fungus was grown on potato-dextrose agar for 7 days at 25 °C. The fresh fungal mycelia of 10 mg were harvested from the plates and total genomic DNA was extracted according to the methods of Doyle and Doyle (1987). Mycelia were grounded with 200 mg of sterilized quartz sand and 600 µl of 2 × CTAB extraction buffer at 65 °C in a 1.5 ml eppendorf tube. The whole contents were incubated at 65 °C water bath for 60 min with swirling once every 10 min. The solution was extracted two or three times

with an equal volume of phenol/chloroform (1:1) at 14,000 rpm for 10 min until no interface was visible. Two volumes of absolute ethanol were added to precipitate the DNA's upper aqueous phase and stored at − 20 °C overnight. The precipitated DNA was washed with 75% ethanol three times, dried under vacuum, suspended in TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8) and stored at − 20 °C.

Three loci were amplified and sequenced for comparisons, including the internal transcribed spacer regions, interval 5.8 S nrRNA gene (*ITS*), beta-tubulin (*tub2*) and translational elongation factors (*TEF1*) gene. *ITS* translation was amplified with forward primer 5′-TCCGCAGGTGAACCTGTAG-3′ (ITS1) and reverse primer 5′-TCCTCCGCTTATTGATCCGC-3′ (ITS4) (White et al. 1990). The forward primer 5′-AACATGCGTGAGATTGTAAGT-3′ (TUB1) and reverse primer 5′-TCTGGATGTTGTTGGGAATCC-3′ (TUB22) were used for amplifying *tub2* gene (O'Donnell and Cigelnik 1997). Part of *TEF1* gene was amplified by primer pairs 5′-ATGGGTAAGGARGACAAGAC-3′ (EF1T) and 5′-ACHGTRCCRATACCACCSATCTT-3′ (1567R) (Rehner and Buckley 2005). PCR was performed in a 25 µl solution containing 1 µl genomic DNA, 12.5 µl 2 × Es Taq MasterMix (Cwbio, Beijing) and 1 µl each primer and the thermal cycling program was as follows: 3 min initial denaturation at 95 °C, followed by 39 cycles of 30 s denaturation at 94 °C, 40 s annealing at 55 °C for *ITS* primers and at 54 °C for *tub2* and *TEF1* genes primers, respectively, 1 min extension at 72 °C, and a final 10 min extension at 72 °C. Eight microliters of PCR products from each PCR were examined by electrophoresis at 100 V for 1 h in a 1% agarose gel and visualized with UV light after staining with ethidium bromide. PCR products were purified using the PCR Purification Kit (Bio-Teke, Beijing) according to the manufacturer's protocol and sequenced with the above primers at Sangon Biotech, then revised sequences were deposed in GenBank database.

### Sequence alignment and phylogenetic analyses

The generated DNA sequences were edited using bioedit software (version 7.2) and the known species of *Pestalotiopsis* were retrieved from the NCBI GenBank database for comparison. Sequences of *ITS* and *TEF1* genes were from 19 taxa (18 *Pestalotiopsis* isolates and 1 *Stemphylium vesicarium*). The *ITS* sequence of *S. vesicarium* (GenBank Accession No. MH861935) and one *tub2* gene sequence of *S. vesicarium* (GenBank Accession No. AY749032) were used for the outgroup in phylogenic analyses of *ITS* and *tub2* genes, respectively. Manual editing of sequences was performed in MEGA6 (version 6.0) (Tamura et al. 2013). The *ITS* and the *tub2* genes were analyzed phylogenetically using the method of Maximum Likelihood (ML). Sequence alignments were conducted using ClustalW program with the default settings. Gaps were introduced to improve alignments. Phylogenetic trees were built and viewed in MEGA 6 with the ML method. All characters were treated as unordered and equally weighted. The branch supports of ML analyses were evaluated using a bootstrapping (BS) method of 1000 replicates (Hillis and Bull 1993).

## Results

### Disease symptoms and pathogen isolation

Disease symptoms on the *P. bungeana* consisted of necrotic lesions at early stage and gummosis at the twig at middle stage (Fig. 1A), twigs above the lesion were died at the end. Among the 6 sampling sites, the average incidence of twig blight reached 92%. 80% shoots in gardens and 72% shoots in plantations died in next spring in 2017, and approximately 72% new twigs were still infected in the field investigation at 2018.

A total of 120 fungal isolates were obtained from diseased *P. bungeana* twigs. Among them, 102 isolates (85.0%) were identified as *Pestalotiopsis* spp. under microscopy, 9 (7.5%) were *Fusarium* spp., 6 (5.0%) were *Trichothecium* spp., while the remaining 3 belonged to *Penicillium* or unknown fungal species.

### Pathogenicity of the isolates

Results of pathogenicity tests revealed *Pestalotiopsis* spp. was pathogenic for *P. bungeana* twigs and induced the symptoms similar to those occurring in the field. The wounded *P. bungeana* twigs initially developed small, circular, ash-coloured lesions, which later changed into brown spots. After 12 days of incubation, the lesion prolonged to 5 mm diam., and

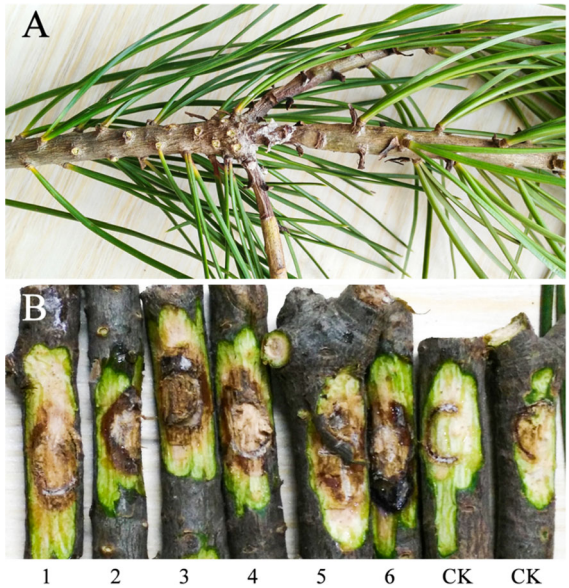



**Fig. 1** **A** Common symptoms of the *Pinus bungeana* blight disease observed in the field. **B** Pathogenicity tests of *Pestalotiopsis* spp. on *P. bungeana* in indoor environments. No disease symptoms were observed on the control *P. bungeana* twigs inoculated with PDA plugs without mycelia and conidia. However, the blight symptoms occurred on *P. bungeana* twigs inoculated with the PDA plugs (1 to 2 $mm^2$) with mycelia and conidia of *Pestalotiopsis* spp. in 1–6

became sunken causing twig blight (Fig. 1B 1–6). In contrast, symptoms were not observed on non-wounded and the controlled twigs, signifying that wounding may be essential for symptom development (Fig. 1B CK). The other isolates of *Fusarium* spp., *Trichothecium* spp., *Penicillium* and the unknown fungal species didn't cause twig blight disease symptoms. In addition, *Pestalotiopsis* spp. can be re-isolated from the wounded inoculation sites at a 100% frequency, and its morphological features and gene sequences were equal to the original ones, which demonstrates that *Pestalotiopsis* spp. is the causal agent for *P. bungeana* twig blight.

## Characterization of *Pestalotiopsis* isolate

The colony on the PDA reached 70 mm in diameter after 7 days at 25 °C, with a full edge, pale honey-coloured, dense aerial mycelia on the surface, black colony conidia, and pale honey at the reverse side (Fig. 2A–C). Most isolates produced conidia that were fusiformed, fusoid, straight to slightly curved, 4-septate, 21.8–28.8 × 5.5–8.5 µm (average

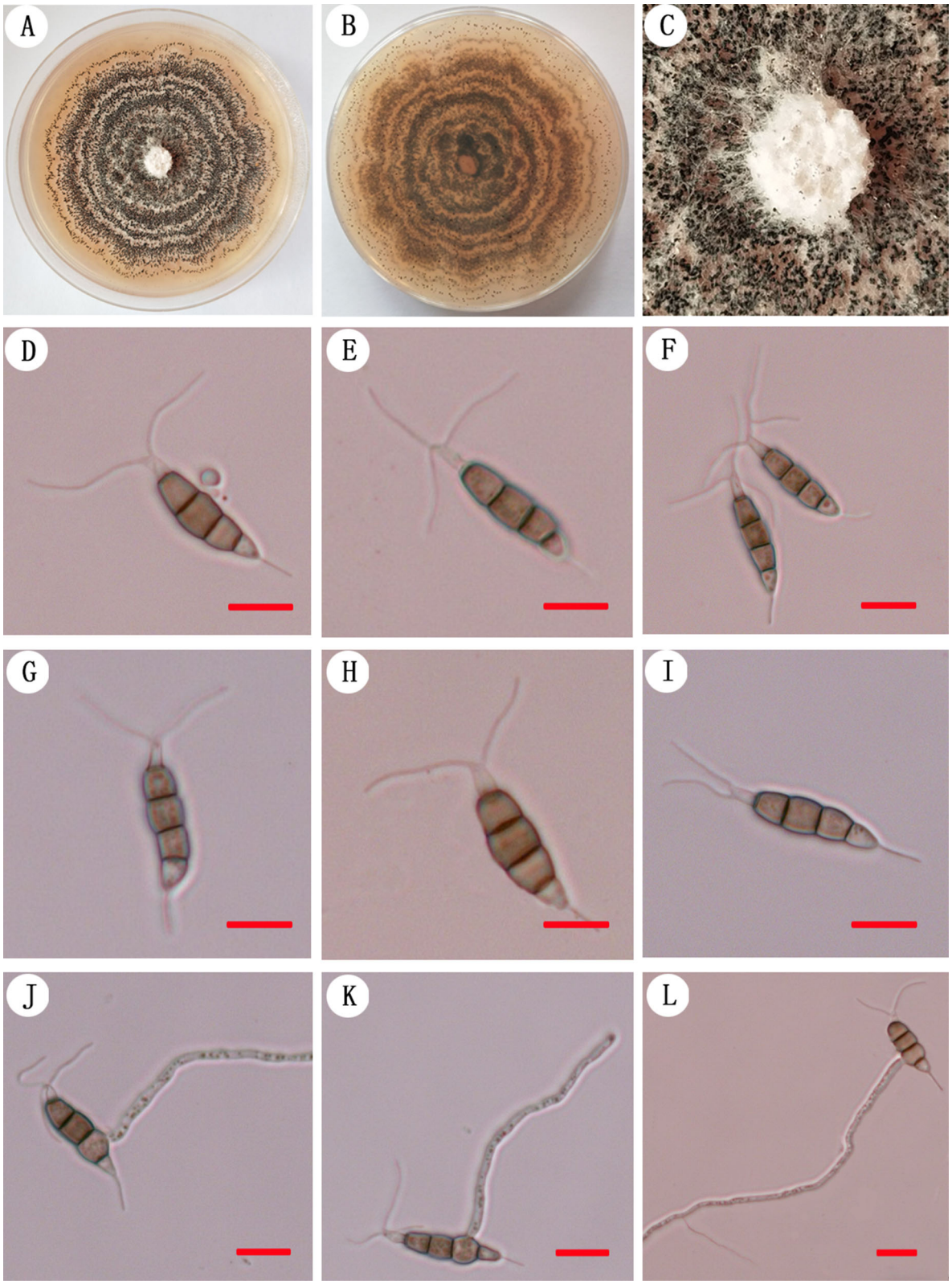
**Fig. 2** Morphology of *Pestalotiopsis trachicarpicola*. *Note* **A.**▶ **C**. Colony on PDA from front side, **B** Colony on PDA from revere side, **D–L** Conidia, **J–L** Germinated Conidia. Scale bars = 10 µm

26.0 ± 1.7 × 7.3 ± 0.7 µm). Basal cell was conic with a truncate base, hyaline, rugose and thin-walled, 3.1–6.9 µm (average 5.1 ± 1.1 µm). The three median cells were coliformed and almost concolourous, pale brown, with the middle one being the darkest and the lower one being the lightest, whose septum was darker than the rest. The upper cell length ranged from 4.5–6.5 µm (average 5.8 ± 0.4 µm), the middle cell length ranged from 4.5–6.5 µm (average 5.6 ± 0.6 µm), and the lower cell length ranged from 4.7–6.7 µm (average 5.8 ± 0.5 µm). Spores germinated from the lower one, and germtubes are hyphal without appresorium at the epic (Fig. 2J–L). The length of apical cell ranged from 2.8–5.5 µm (average 3.7 ± 0.7 µm), hyaline, obconic with a truncate base or subcylindrical, thin-walled, with 2–3 tubular apical appendages (mostly 3), arising from the apical crest, unbranched (rarely branched), filiformed, 9.8–25.3 µm (average 16.9 ± 3.7 µm). Basal appendage was single, tubular, unbranched, centric, length ranged from 3.6–8.2 µm (average 6.6 ± 1.2 µm) (Fig. 2D–I). Morphology of each *Pestalotiopsis* spp. isolate under microcopy is almost identical.

## Phylogenic study

Phylogenetic trees were constructed using individual *ITS* and *tub2* genes sequences of 19 *Pestalotiopsis* isolates and a *S. vesicarium* species as the outgroup taxon. The size of the *ITS* region, including *ITS1*, *ITS2* and the 5.8S rRNA gene, was 540 base pairs (bps) for isolate BAI-Xp (GenBank Accession No. MN955320), one high frequent isolate from diseased shoots in the field. Based on the phylogenetic tree in Fig. 3, isolate BAI-Xp, grouped with *Pestalotiopsis* species known to possess median cells that range in colour from yellow ocher to tawny-olive, concolourous or almost concolourous (Wei et al. 2005; Ge et al. 2009), formed a complex clade with *P. trachicarpicola* and *P. neglecta* (PP = 0.72, Fig. 3).

*Tub2* gene sequence of isolate BAI-Xp (GenBank Accession No. MN947739) was 759 bps. Phylogenetic analysis revealed that it was a monophyletic group of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*P. trachicarpicola*, which was different from the other tested *Pestalotiopsis* species at bootstrap 100% (Fig. 4).

*TEF1* gene sequence of isolate BAI-Xp (GenBank Accession No. MN947738) was 494 bps, when aligned with sequences in the GenBank, the *TEF1* fragment of isolate BAI-Xp showed a 99% coverage and 100% homology with *P. trachicarpicola* strain GBLZ16PE-005 and a 92% coverage and 100% homology with *P. neglecta* strain TAP99M112, but there are only these two species with *TEF1* sequences in GenBank database. Therefore, no phylogenic tree on *TEF1* gene was constructed in this study.

## Discussion

The genus *Pestalotiopsis* was established by Steyaert (1949) based on the typical species produced relatively fusiformed conidia with 3 coloured median cells and 2 colourless end cells as well as two or more apical appendages. The interspecific division of the genus *Pestalotiopsis* is mainly based on morphological characteristics of conidia, conidiogenesis, teleomorph, and host associations (Steyaert 1949; Benjamin and Guba 1960; Sutton 1980; Zhu et al. 1991; Ge et al. 2009). Among them, the pigmentation of median cells is a reliable character in the classification of *Pestalotiopsis* species (Griffiths and Swart 1974; Hu et al. 2007; Liu et al. 2010). It is between ''brown concolourous" and ''versicolourous". Presence or absence of basal appendages and apical appendage can be used as additional taxonomic characters for defining *Pestalotiopsis* species (Jeewon et al. 2003; Li et al. 2020). The novel pathogen in this study, initially designated as concolourous, was identified mainly based on conidial morphology using the above rules. Therefore, we downloaded 19 sequences of *Pestalotiopsis* with concolourous brown median cells from GenBank and assigned this cryptic fungus as *Pestalotiopsis trachicarpicola* in this study.

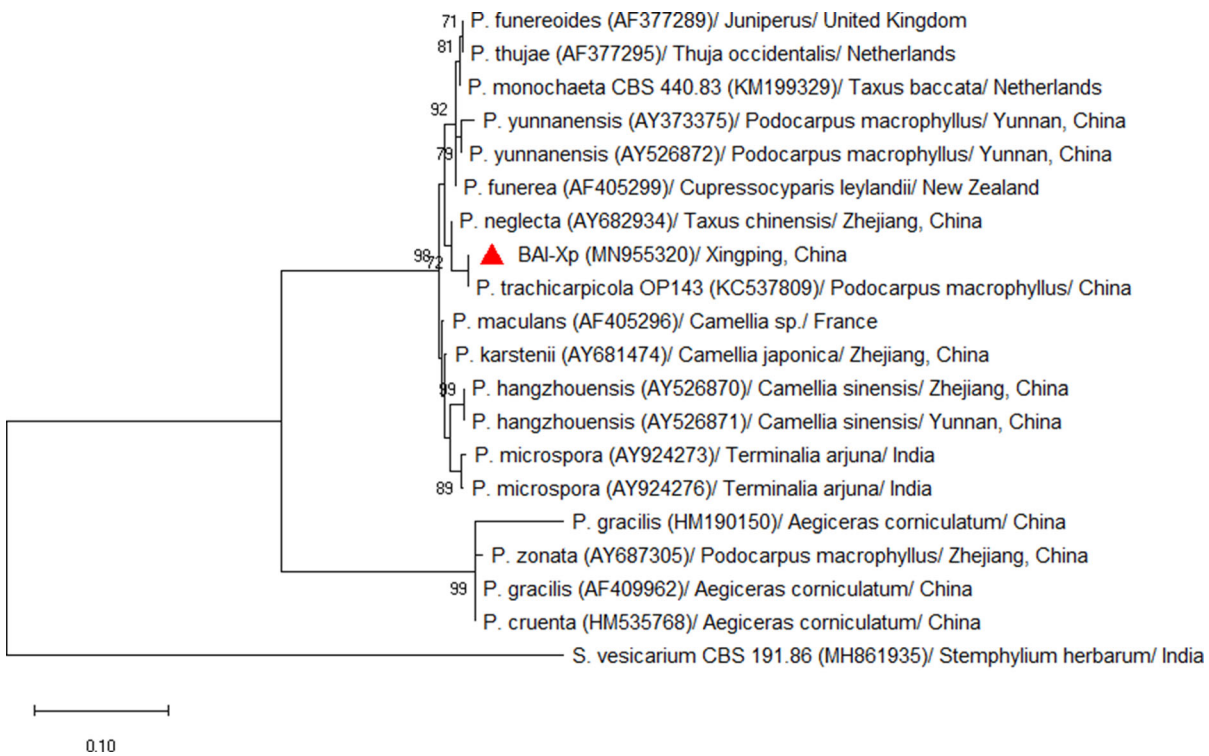


**Fig. 3** Phylogenic tree of *ITS*-DNA sequences based on Maximum Likelihood method. *Note* The number at each branch indicated the bootstrap value in 1000 replications. Numbers above the twigs were presented as bootstrap values greater than 50%. The GenBank accession number was indicated in the parenthesis and the information on host and geographic origins were attached thereafter. The new report species was shown in red triangles

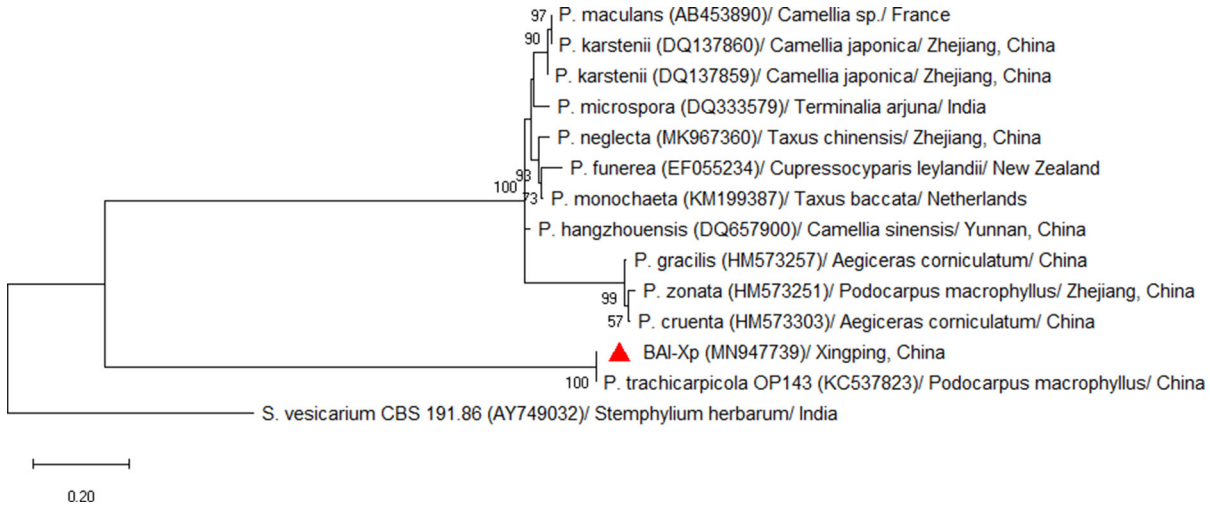


**Fig. 4** Phylogram of *tub2* gene sequences based on Maximum Likelihood method. *Note* Numbers above the twigs were presented as bootstrap values greater than 50%. The GenBank accession number was indicated in the parenthesis and the information on host and geographic origins were attached thereafter. The new report species was shown in red triangles

Jeewon et al. (2002) proposed that *Pestalotiopsis* is a monophyletic genus through molecular studies. In the genus, almost 249 species of *Pestalotiopsis* have been reported (CABI Bioscience Database 2012). After analyzing a combined dataset of DNA sequences for the *ITS* region and the gene encoding *tub2*, Liu et al. (2010) found that *tub2* gene can phylogenetically substantiate the findings.

We analyzed sequence data from two gene regions respectively to evaluate their ability to resolve species limits of *Pestalotiopsis* of this novel disease. The *ITS* DNA is the universal barcode for fungi classification at the taxonomical level of family and genus (Schoch et al. 2012) with two diverse *ITS* sequences and two exons encoding a conserved ribosomal protein (Bridge et al. 2005; Schoch et al. 2012). *ITS*-DNA fragments can be yielded by PCR amplification successfully from the six *Pestalotiopsis* isolates, and showed a high conservative sequencing. However, the *ITS* sequence is still fragile to be a candidate gene for discrimination of the cryptic species, since it did not involve diverse variation among species. Thus, we employed another orthologous gene, *tub2*, to discriminate this new disease pathogen, which was composed of better resolution for most cryptic species than ribosomal regions (Schoch et al. 2009; Hu et al. 2007). *tub2* gene is a widely used taxonomic marker in *Pestalotiopsis* and has been successfully utilized to resolve species in other genera in groups (Roger et al. 1999; Baldauf et al. 2000), such as *Seiridium* (Barnes et al. 2001), *Discosia* and *Seimatosporium* (Tanaka et al. 2011). In the *ITS* phylogenetic tree (Fig. 3), *P. trachicarpicola* cannot be differentiated from *P. neglecta*, but *tub2* phylogenetic tree can differentiate it from all other *Pestalotiopsis* species. Isolated BAI-Xp and *P. trachicarpicola* were nested within an independent phylogenetic clade. Another orthologous gene, elongation factor 1-alpha (GenBank Accession No. MN947738), was also employed for limiting this *Pestalotiopsis* isolate, and when aligned with other species in the GenBank, it also showed 99% coverage and 100% homogenesis with sequences of *P. trachicarpicola.*

*P. trachicarpicola* has been recorded from *Trachycarpus fortunei* (Chinese windmill palm), *Chrysophullum* sp. (Rare star Apple), Schima sp., Symplocos sp., Mango and grapevine in China (Maharachchikumbura et al. 2013; Zhang et al. 2012; Jayawardena et al. 2016), but the infected organisms are all leaves, and the microscopic describes for this pathogen from above trees are nearly identical with the isolate BAI-Xp. To our knowledge, this is the first description of *P. trachicarpicola* as causal agents for twig blight on *P. bungeana* in China.

## Conclusion

This study represents the first attempt to identify and characterize the *Pestalotiopsis*-like fungi causing twig blight in *P. bungeana* by using both morphological and molecular approaches. This is the first report of *Pestalotiopsis trachicarpicola* causing diseases in *P. bungeana*.

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**Author's contribution** ZDY conceived the idea and designed the experiments. MQ and CXX carried out the research experiments and analyzed the results. MQ and ZDY drafted the manuscript. ZDY and QWC revised the manuscript. All authors read and approved the final version of the manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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

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