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ORIGINAL ARTICLE

Isolation and identification of *Esteya vermicola* and its potential for controlling pinewood nematode

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Abstract

Pine wilt disease is a devastating forest disease worldwide caused by the pinewood nematode (PWN; *Bursaphelenchus xylophilus*). *Esteya vermicola* is widely recognized as a promising bio-control agent effective against PWN. During a survey of associated microorganisms of *Pinus yunnanensis* in Southwest China, a novel isolate of *E. vermicola* was obtained. This isolate produced two types of conidia. One type of conidia was hyaline, unicellular, bacilloid, and non-adhesive. The second type of conidia is solitary, hyaline, unicellular asymmetric elliptic or lunate, concave inward, ending slightly apiculate, adhesive on the concave surface, and contained an ovoid endospore-like structure. Only the lunate conidia showed infection activity against PWN. This novel isolate produced a high proportion of infective lunate conidia and exhibited high adhesion and infection activity against PWN by adhering to the nematode within 48 h and killing 74.5% of the tested PWN individuals within 4 days. Given that the isolate was obtained from *P. yunnanensis*, it may be well adapted to the endogenous environment of pine trees. The isolate shows potential as a bio-control agent against a plant-parasitic nematode in the field.

KEYWORDS
biological control and prevention, *Bursaphelenchus xylophilus*, *Esteya vermicola*, Nematophagous fungus, pine wilt disease

INTRODUCTION

Pine wilt disease is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934) Nickle 1970 (Nickle et al., 1981). *Monochamus* Dejean 1821 spp. beetles (Morimoto, 1972) are the major vectors for *B. xylophilus* infection and *Pinus* L. spp. are the predominant hosts (Enda & Mamiya, 1972; Kiyohara & Tokushige, 1971). *Bursaphelenchus xylophilus* was detected in Japan in the early twentieth century and quickly spread to China (Sun, 1982), South Korea (Han et al., 2008), and subsequently to European countries such as Portugal (Mota et al., 1999) and Spain (Abelleira et al., 2011). The disease causes severe economic losses in forests of several countries in the world. Given the economic and environmental impacts of the disease, increasing research attention is focused on the prevention and control of pine wilt disease.

Esteya vermicola Liou et al. (1999) was the first reported endoparasitic fungus of PWN. This fungus is highly infectious of PWN and exhibits great potential as a bio-control agent against pine wilt disease (Liou et al., 1999). The species is characterized by the production of two types of conidia: lunate conidia and bacilloid conidia. When the nematode comes into contact with the lunate conidia, the conidia adhere to the nematode body. The conidia germinate, penetrate the nematode cuticle by means of a penetration peg, and subsequently hyphae are initiated. The hyphae colonize the internal tissues of the nematode and produce adhesive flask-shaped conidiogenous cells and lunate conidia. The fungus destroys the organs

and tissues, and the nematode is killed typically within 4 days (Du et al., 2014; Wang et al., 2008).

Previous studies have investigated the potential of *E. vermicola* as a bio-control agent against pine wilt disease in inoculation experiments. *E. vermicola* can survive in the resin of pine without causing necrosis or discolouration (Wang et al., 2011), and successfully infects PWN individuals within inoculated trees (Sung et al., 2010). The infection cycle consists of the growth of *E. vermicola*, attraction and adhesion to PWNs, movement and death of infected PWNs, production of a second generation of lunate conidia, and attraction of other uninfected PWNs within the inoculated trees (Yin et al., 2020).

Up to now, a total of eight strains of *Esteya* have been reported. The *E. vermicola* was first reported from Taiwan, China (Liou et al., 1999). Its taxonomy and parasitism of *B. xylophilus* have been reported in detail. The fungus was next isolated from the surface of larvae and adult beetles of *Scolytus intricatus* Ratzeburg 1837 and their galleries under the bark of branches from three species of oak (*Quercus* L.) in the Czech Republic. Subsequently, one isolate was isolated from infected nematodes in forest soil samples in Korea (Wang et al., 2008). In the following year, Wang et al. (2009) researched the morphological characteristics and infection activities against *B. xylophilus* of two isolates of *E. vermicola*, CBS 156.82 and CBS 100821, maintained at CBS (National Collection of Type Cultures, Netherlands) that were isolated from Japan and Italy respectively (Wang et al., 2008). The CBS 156.82 was isolated from dry *Pinus* in 1982 while the CBS 100821 was isolated from olive twig in 1998 (Mao et al., 2020). An additional isolate of *E. vermicola* was isolated from *Bursaphelenchus rainulfi* Braasch & Burgermeister, 2002 which intercepted from wood packaging materials originating in Brazil and arriving at Tianjin port in China. Wang et al. (2019) isolated another novel isolate of *E. vermicola* from *Tomicus yunnanensis* Kirkendall and Faccoli 2008 and its host *Pinus yunnanensis* Franch.1899 in Yunnan, China. In addition, Li et al. (2018, 2020) isolated a fungus of the genus *Esteya* from the head of *Myoplatypus flavicornis* Fabricius 1776 from loblolly pine (*P. taeda* L.) in Florida, USA. Based on phylogenetic analysis of β-tubulin and 28S large subunit sequences and morphological characteristics, the isolate was identified as a species of *Esteya* and was named *E. floridanum* Y. Li, Araújo & Hulcr 2018 (Li et al., 2018, 2021). This fungus was observed to kill PWN in vitro and delayed the symptoms of pine wilt disease.

During a survey of microorganisms associated with *P. yunnanensis* in southwest China, a novel isolate of *E. vermicola* was isolated and identified based on its morphological and molecular characteristics. The infection activity of the fungal isolate against PWN, including the attraction and adhesion to PWNs, was investigated.

MATERIALS AND METHODS

Isolation of fungi

In total, 81 samples from 15 to 30-year-old *P. yunnanensis* in artificial forest, comprising 45 samples from Kunming and 36 samples from Dali, Yunnan Province, China, were collected and used in this study (Table 1). For each sample, 2–3-year-old branches (more than three individuals), and main stems (upper, middle and lower sections), were collected for isolation of fungi. The stems (approximately 5 × 5 × 3 cm in longitudinal, radial, and tangential directions, including the whole bark, phloem, cambium, and a few xylem rings [1–2 cm]) were cut from trunk. The branches were cut into discs of approximately 3 cm thickness. The branches and stems from the same tree were collected as one sample and were immediately placed in plastic bags to prevent desiccation and then were transported to the laboratory. The sample surface was removed from any dust by dry sterile tissue and then scraped to remove the dead tissue using a sterile razor blade. The tissue isolation and hyphal-purification method was used to isolate and purify fungi associated with the samples (Wang et al., 2019). Briefly, all tissue blocks were disinfected with NaClO solution (0.5%, w/v) for 1 min, further surface-sterilized with 75% ethanol for 1 min, and rinsed in sterile water three times for 1 min each. After carefully removing moisture on the surface with absorbent paper, the tissue block was divided into xylem part and bark part (including bark, phloem and cambium). All tissues were sliced into 5 mm × 5 mm × 1 mm sections. All tissue sections were cultured on potato dextrose agar (PDA; Nissui-seiyaku) and water agar medium plates at 25°C in the dark. Small mycelial plugs (5 mm) were selected from an area of active mycelial growth near the edge of a 2- to 5-day-old culture, placed on PDA, and incubated at 25°C in the dark for 7 days. Pure cultures were stored at −4°C in 10% sterile aqueous glycerol solution and used for subsequent research.

Identification

The morphological and cultural characteristics of the colony were observed during incubation on PDA medium for 7 days in the dark

TABLE 1 The information of collection sample

Origin	Location	Information of host
Kun Ming (An Ning)	E:102°50′13″N:25°11′53″	6 asymptomatic, 6 Infected[a]
Kun Ming (Pan Long)	E:102°50′18″N:25°12′44″	6 asymptomatic, 6 Infected[a]
Da Li (Tian Feng mountain)	E:100°53′56″N:25°18′46″	6 asymptomatic, 8 Infected[a]
Da Li (Leng Shui Jing)	E:100°55′N:25°19′45″	3 asymptomatic, 4 Infected[a]

[a]Asymptomatic, asymptomatic *Pinus yunnanensis* without any symptom; Infected, infested *P. yunnanensis* by *Tomicus yunnanensis* with wilt (showed in Figure 1).

at 25°C. Colony colour (surface and reverse) was rated based on the colour charts of Rayner (1970). Other morphological characteristics of the fungus, including the mycelium, conidiophores, and conidia, were examined with a light microscope (Axio Imager A2, Zeiss). Lactophenol cotton blue stain was added to the slide in order to examine the conidia structure. Measurements of the conidiophores and conidia were determined by microscopic examination of at least 100 replicates. The ANOVA of all statistical data were analysed using IBM SPSS Statistics 19.0 software (IBM Corporation). Descriptive statistics were calculated by the formula of (minimum–) (mean–standard deviation)-(mean + standard deviation) (–maximum).

Mycelia and conidia were collected by scraping with a sterile blade the medium surface of cultures grown on PDA at 25°C in the dark. The samples were placed in a 1.5 ml sterile tube. Total genomic DNA was extracted using the Plant Genomic DNA Kit (Tiangen). The β-tubulin and elongation factor-1α gene fragment were amplified by PCR using the genomic DNA as the template, the synthetic primers (β-tubulin forward primer, bt2a 5′-GGTAACCAAATCGGTGCTGCTTTC-3′ and reverse primer, bt2b 5′-ACCCTCAGTGTAGTGACCCTTGGC-3′ (Glass & Donaldson, 1995), elongation factor-1α forward primer, ef-f 5′-TGCGGTGGTATCGACAAGCGT-3′ and reverse primer, ef-r 5′-AGCATGTTGTCGCCGTTGAAG-3′ (Jacobs et al., 2004) and 2× *Taq* PCR MasterMix (Tiangen). Each PCR amplification was performed using 2 μl primers (10 μM), 25 μl of 2× *Taq* PCR MasterMix (0.1 U Taq Polymerase/μl, 500 μM dNTP each, 20 mM Tris–HCl [pH 8.3], 100 mM KCl, 3 mM $MgCl_2$), 21 μl ddH_2O, and 2 μl DNA template (0.02 μg/μl). The PCR thermal cycling protocol was initial denaturation at 94 °C for 4 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a single extension cycle of 72°C for 10 min. The amplified products were detected by 1.0% agarose gel electrophoresis and used for direct PCR sequencing, which was performed by BGI Biology Co. Sequences were used for a BLAST search of the National Center for Biotechnology Information databases (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species of pure cultures and then submitted to the NCBI database. The phylogenetic analysis included the novel isolate in this study and reference isolates representing *E. vermicola* and other ophiostomatalean fungi related to *Esteya* (Wang et al., 2014). The β-tubulin and elongation factor-1α nucleotide sequences of closely related species were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) to construct phylogenetic tree. The nucleotide sequences information used for phylogenetic analysis is shown in Table 2. Alignments of novel sequence and related sequences were made using MAFFT 7.0 (Katoh & Standley, 2013) (https://mafft.cbrc.jp/alignment/server/index.html/). Phylogenetic analyses were performed using maximum likelihood (ML). ML analyses were implemented online using PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/ under the GTR-GAMMA model. Support for the nodes was estimated from 1000 bootstrap replicates. Bayesian analyses were performed using MrBayes 3.2.6. Bayesian posterior probabilities (BPP) were estimated with two chains executed simultaneously for 1,000,000 generations, sampling every 200 generations, and a burn-in of the first 100,000 trees. The phylogenetic tree was exported and manually adjusted by Figtree v.1.4.0.

Adhesion and nematocidal tests

To investigate the infection of PWN, the conidia proportion of pure culture was verified after incubation on PDA medium for 10 days in the dark at 25°C. The fungal mats were scraped with sterile spreader in sterile water and then washed five times with sterile water until the wash solution contained no conidia. The wash solution was volume to 15 ml and counted the number of conidia using the haemocytometer. Ten biological replicates were performed. *Bursaphelenchus xylophilus* isolate NXY61 used in this study was isolated from infected wood chips of *P. massoniana* Lamb. in Ningbo, Zhejiang Province, China, and maintained in the Forest Pathogen Integrated Biology Laboratory of the Chinese Academy of Forestry, Beijing, China. *Botrytis cinerea* Pers. isolate used in this study was also maintained in our laboratory. Nematodes were cultured on fungal mats of *B. cinerea* grown on PDA plates at 25°C for 8 days. Nematodes were collected using the Baermann funnel technique. The *E. vermicola* was cultured on PDA medium (60 mm) at 25°C for 10 days. And then a volume of 20 μl of mix stage population of PWN suspension (~1000 nematodes) was inoculated on the fungal mats of *E. vermicola* and incubated at 25°C. Nematodes were extracted at 6, 12, 24, 48, and 96 h after inoculation. The fungal mats inoculated with PWN were soaked in sterile water for 1 h, then washed five times with sterile 1× phosphate buffered saline with Tween 20 until the wash solution contained no nematodes. The adhesion and mortality of nematodes were determined by observation at ×10 magnification using an inverted phase-contrast microscope (Primovert, Zeiss). Adhesion was calculated as the percentage of nematodes with adhered conidia among the total number of nematodes. The mortality was calculated as the percentage of dead nematodes among the total number of nematodes. Nematode death was determined by the lack of response to physical stimuli. Ten biological replicates were performed at each time point. The ANOVA of all statistical data was analysed using IBM SPSS Statistics 19.0 software (IBM Corporation). All data were expressed as the mean and standard deviation.

Potential assay for controlling PWN

In order to further research the nematicidal activity of *E. vermicola* and potential for controlling PWN, we have performed the inoculation experiment at Yongkang, Zhejiang Province, China from June to August 2020. Thirty healthy *P. massoniana* (15–20 years old, 10–15 cm diameter at breast height) were selected and randomly divided into three groups. Group EV was the treatment

TABLE 2 Species and origin of strains included in this study

Species	Strain no.	Location	GenBank accession no.	
			BT	EF
[a]	Fxy121	China	MT323207	OK500296
Esteya vermicola	CBS115803	Czech Republic	FJ490552	GQ995672
E. vermicola	CNU120806	Korea	FJ490553	GQ995673
E. vermicola	NKF132229	Brazil	KC831752	–
E. vermicola	Cxy1893	China	MH697597	MH605999
E. vermicola	CBS 100821	Korea	–	GQ995671
Ambrosiella macrospora	CBS367	Canada	EU977465	–
A. tingens	CBS366	Canada	EU977468	–
Grosmannia huntii	JKM58	New Zealand	JQ918165	–
G. huntii	JKM72	New Zealand	JQ918169	–
G. huntii	VPRI43837	Australia	–	MW066410
G. koreana	MCC214	Japan	AB222064	–
G. koreana	MCC206	Japan	AB222063	–
G. koreana	CMW39395	USA	-	KF515888
G. piceaperda	CMW448	USA	JF280025	JF280079
G. piceiperda	RAE6D-3-21-Gp	Canada	FJ269188	–
G. piceiperda	CMW3314	Australia	–	JF280075
Graphilbum anningense	CXY1900	China	–	MH606001
Leptographium koreanum	MUCL46335	China	EU502810	EU502825
L. koreanum	MUCL46362	China	EU502811	EU502826
L. lundbergii	CMW217	South Korea	AY707185	DQ062032
L. truncatum	NZFS169B	New Zealand	JQ013497	–
L. truncatum	MUCL46453	China	EU502808	EU502823
L. yunnanense	CMW5304	Korea	AY707192	AY536209
L. yunnanense	CMW5152	Korea	AY707193	DQ062040
Ophiostoma breviusculum	DUCC3002	South Korea	JQ886725	–
O. piceae	CMW8093	Canada	DQ296091	–
O. piceae	KUC 2015	New Zealand	AY789150	–
O. piceae	YCC-731	Japan	–	LC090742
O. piceae	H2154	Japan	–	AB934352
O. piceae	AU100-1	Japan	–	AB934350
O. canum	NFRI 1652/2	Netherlands	JQ886733	–
O. canum	CMW 23261	Russia	–	KF899869
O. canum	YMF1.04967	China	–	MH195221
O. pseudotsugae	OM5	UK	AY542510	–
O. floccosum	DKM 0514	South Korea	FJ603433	–
O. floccosum	Gr1'0	Canada	–	KF899873
O. floccosum	CMW:34182	China	–	KU184388
Pesotum aureum	MUCL46632	China	EU502819	–
Sporothrix inflata	CMW12527	Germany	DQ296075	–
S. inflata	CMW12531	Chile	AY495440	–
S. chilensis	Ss469	Chile	–	KP711817
S. schenckii	CMW2429	South Africa	EF139106	–
S. schenckii	CMW7614	South Africa	AY280477	–

TABLE 2 (Continued)

Species	Strain no.	Location	GenBank accession no.	
			BT	EF
S. schenckii	CBS 132979	Peru	–	KJ002349
S. luriei	CBS 937.72	South Africa	–	KC576615
S. Mexicana	CBS 120341	South Africa	–	KC576611
S. brasiliensis	CBS 120339	Netherlands	–	KP016994
S. schenckii	CBS 359.36	South Africa	–	KC576614
Arthrobotrys oligospora	CBS 337.94	Germany	AY444616	–
A. oligospora	920	China	–	AY773404

[a]Novel strain; Sequences missing data are indicated by [–].

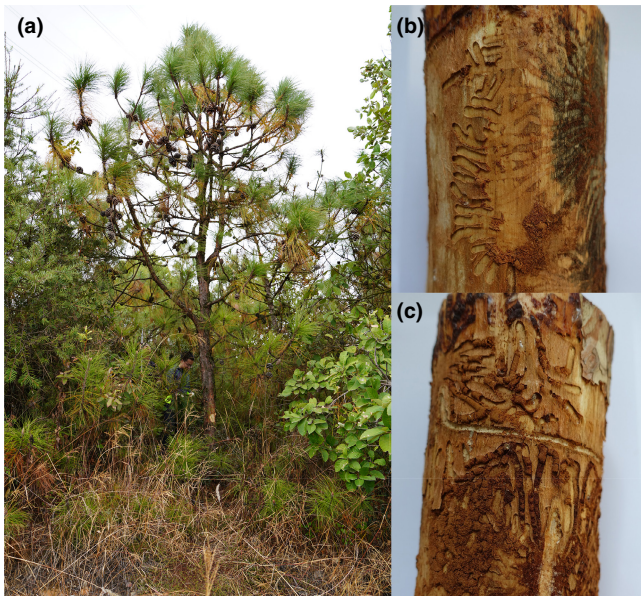

FIGURE 1 The host of *Esteya vermicola*. (a) *Pinus yunnanensis* infected by *Tomicus yunnanensis*; (b, c) the trunk with galleries

group in which trees were injected with 15 ml conidia suspensions (1.0 × 108 conidia/ml) for 30 days and then inoculated with 15 ml PWN suspensions (10,000 nematodes). Trees in group PWN were injected with 15 ml sterile water for 30 days and then inoculated with the same number of nematode suspensions as the infected control (positive control). Group W was all inoculated with sterile water at the same time as the uninfected control (negative control). The inoculation method of *E. vermicola* and PWN was followed by the method reported by Yin et al. (2020). Briefly, one hole was made by drilling tilt by 30°–45° to the trunk of each tree and blocked with plug after injecting the conidia or nematode suspensions. The conidia of *E. vermicola* was washed from PDA mats cultured for 10 days in the dark at 25°C. Nematode was collected by Baermann funnel technique after incubation on PDA for 8 days at 25°C. The inoculation position was shown in Figure S1. Ten biological replicates were performed in every group. The symptoms were observed and recorded after inoculation with nematode for 30 days. Disease symptoms were assessed based on a scale of 0 to 5 as follows (Yu et al., 2012): 0, all needles were green; 1, 0%–20% of the needles turned yellow; 2, 20%–40% of the needles turned yellow; 3, 40%–60% of the needles turned yellow; 4, 60%–80% of the needles turned yellow; 5, 80%–100% of the needles turned yellow. The infection rates and disease severity index (DSI) and relative control effect (RCE) were calculated using the following formulae (Meng et al., 2019; Yu et al., 2012). The infection rates were the proportion of infected trees. The DSI was represented as the disease severity. The RCE analysed the control efficacy of Fxy 121 isolate for PWN.

$$\text{Infection rate} = \frac{\sum \text{Number of infected trees}}{\text{Total number of infected trees}} \times 100\% \quad (1)$$

$$\text{DSI} = \frac{\sum \text{Number of diseased trees} \times \text{symptom grade}}{\text{Total number of diseased trees} \times \text{highest symptom grade}} \times 100 \quad (2)$$

$$\text{RCE}\ (\%) = \frac{\text{DSI of inoculation with PWN} - \text{DSI of inoculation with } E.\ vermicola}{\text{DSI of inoculation with PWN}} \times 100 \quad (3)$$

RESULTS

Morphological characterization

A fungal isolate was obtained from the bark of symptomatic *P. yunnanensis* trunk infested by *T. yunnanensis* (Figure 1a–c) in Xiaohe Township, Panlong District, Kunming City, Yunnan. On PDA medium, the white aerial hyphae grew densely after incubation for 1 day at 25°C and the colonies were dark green after 3 days. The colony radius was 48.6 mm after culture for 8 days at 25°C, with margins smooth, regular, and abundant fluffy aerial mycelium (Figure 2a,b). On PDA medium, two types of conidia were produced. The first type of conidia was hyaline, unicellular, bacilloid, non-adhesive, (3.72–) 4.94–6.85 (–7.58) × (1.38–) 2.03–2.67 (–3.04) μm (Figure 2e). The second type of conidiogenous cells was solitary, upright, flask-shaped, inflated at the base, tapering to the top, and crooked (Figure 2c,d);

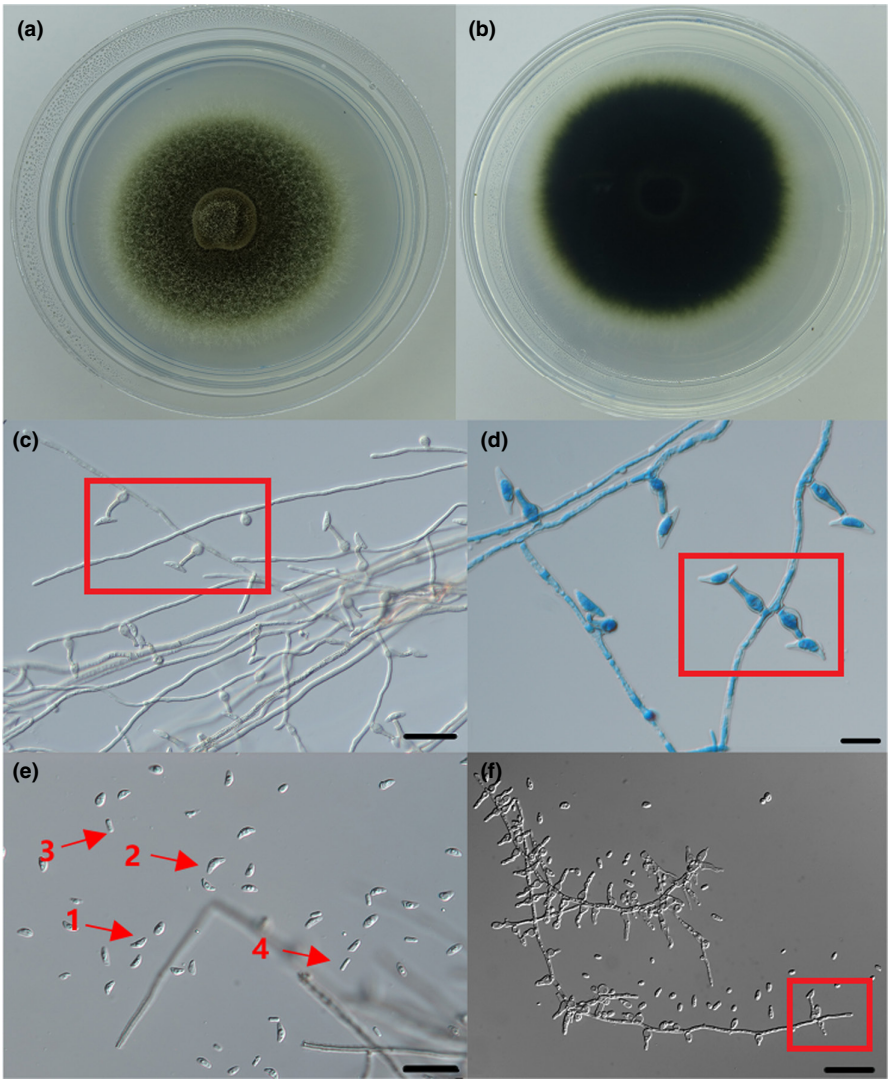

FIGURE 2 The morphological features of *Esteya vermicola*. (a, b) upper and reverse of cultures on PDA 8 days after inoculation; (c, d) conidiogenous cells with lunate conidia (showed in red box); (e) two type conidia (red arrows 1, 2 showed lunate conidia, red arrows 3, 4 showed bacilloid conidia); (f) spore in liquid PDA (conidiogenous cells with lunate conidia in liquid PDA were showed in red box). Scale bars: 100 μm (c); 10 μm (d); 20 μm (e, f)

the conidia were solitary, hyaline, unicellular, asymmetric elliptic or lunate, concave inward, ending slightly apiculate, (6.27–) 7.34–9.94 (–11.65) × (2.90–) 3.15–4.12 (–4.79) μm (Figure 2e), adhesive on the concave surface, and containing an ovoid endospore-like structure (Figure 2e). After culture with shaking in PDB, blastospores were round, oval, or oblong. The blastospores germinated, produced short hyphae, and formed ampoule-shaped conidiogenous cells and lunate conidia (Figure 2f). Based on the aforementioned morphological characteristics, the isolate was identified as *E. vermicola*. The isolate was stored in our laboratory and designated Fxy121, and used in the following research.

Sequence comparisons and phylogenetic analyses

The β-tublin and elongation factor-1α nucleotide sequences of Fxy121 were 413 and 886 bp respectively (Figures S2 and S3). The two sequences were submitted to GenBank (accession nos MT323207 and OK500296). The coverage and identity of the Fxy121 β-tubulin sequence were 100% compared with the sequences for *E. vermicola* isolates NKF13222, CNU120806, and CBS 115803, and sequence identity was 99.75% with the isolate Cxy1893 (Figure S4). The coverage and identity of the Fxy121 elongation factor-1α sequence were 100% compared with the sequences for *E. vermicola* isolates Cxy1893, and sequence identity was 99.65% and 99.76%with the isolate ATCC74485 and NKF13222 respectively (Figure S5). All sequences information for the *E. vermicola* isolates is presented in Table 2. Phylogenetic analyses were obtained from ML and BI of the β-tubulin nucleotide sequence of Ophiostomataceae including *Esteya* with *Arthrobotrys oligospora* as outgroup. Four clades were separately formed by all relative species. Clade A included *Leptographium* spp. and *Grosmannia* spp. Clade B and D comprised *Esteya* spp. and *Sporothrix* spp. respectively. Clade C included several species of *Ophiostoma* spp., *Arthrobotrys* spp. and one strain of *Pesotum aureum*. The isolate Fxy121 was grouped in clade B with high support values (*Esteya vermicola*, 100% BP, 1.00 BPP) (Figure 3). Based on the phylogenetic analyses obtained from ML and BI

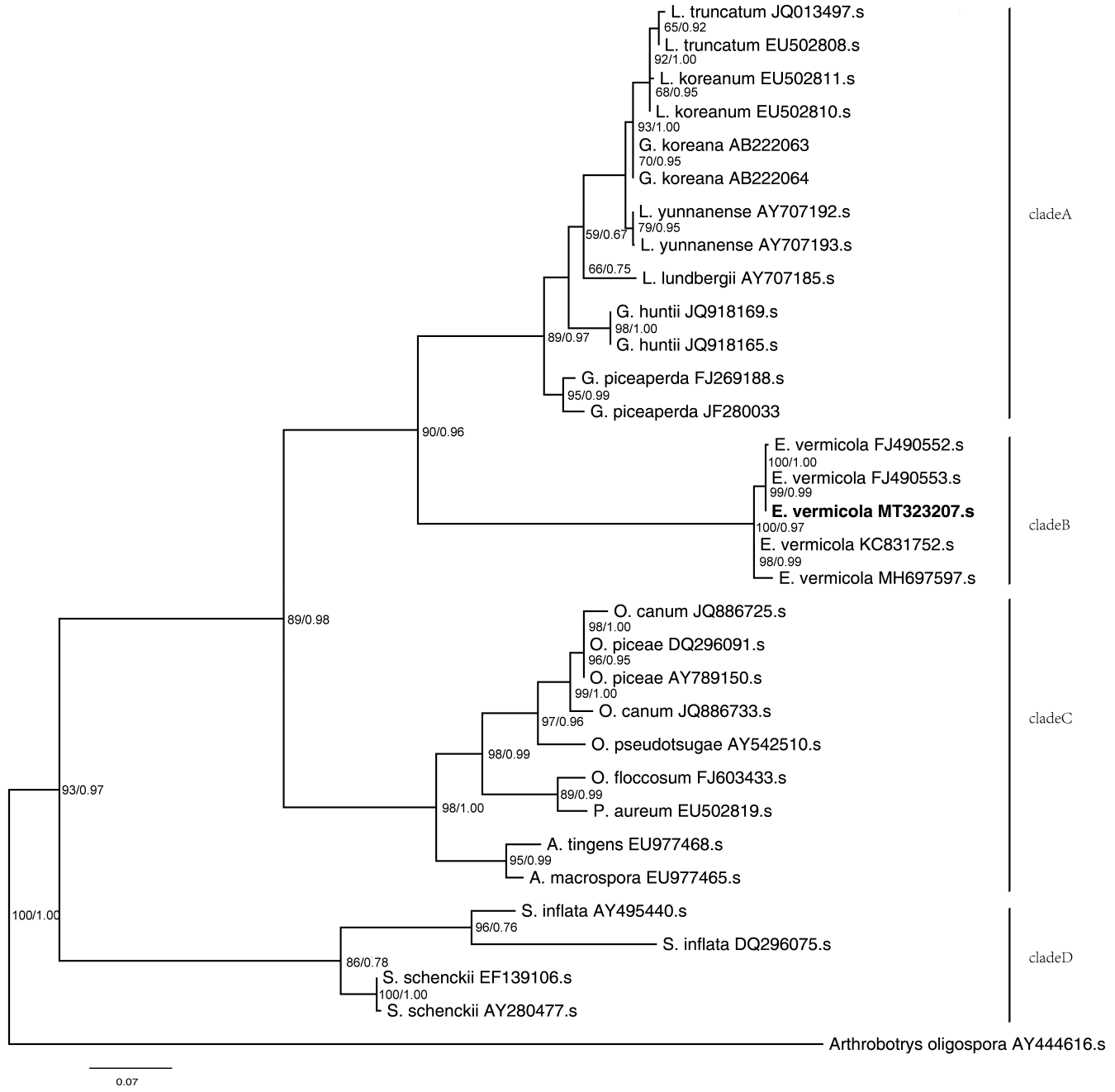

FIGURE 3 Phylogenetic analyses based on ML and BI analysis for *Esteya vermicola* Fxy121 fromβ-tubulin nucleotide fragment. Novel sequence obtained in this study is signed in bold type. Maximum likelihood bootstrap support values((before the/) ≥50% were indicated with numbers. Bayesian posterior probability (BPP) support (after the/) ≥0.60 indicated at the branches. The tree is drawn to scale with branch length measured in the number of substitutions per site. Clade A—*Leptographium* spp. and *Grosmannia* spp., clade B—*Esteya* spp., clade C—*Ophiostoma* spp., *Arthrobotrys* spp. and one strain of *Pesotum aureum*, clade D—*Sporothrix* spp. The isolate Fxy121 grouped in clade B

of the elongation factor-1α nucleotide sequence, three clades were separately formed by all relative species. Clade A included *Leptographium* spp. and *Grosmannia* spp., Clade B included several species of *Ophiostoma* spp. and Clade C comprised *Esteya* spp. and *Sporothrix* spp. The isolate Fxy121 was grouped in clade C with high support values (91% BP, 0.89 BPP) (Figure 4). These results supported the identification of the isolate Fxy121 as *E. vermicola*.

Nematicidal activity

After being cultured on PDA for 10 days at 25°C, Fxy121 of *E. vermicola* produced 90% of infective lunate conidia and 10% of bacilloid conidia (Figure 5) and the concentration of all conidia on the fungal mate was about 1.85×10^7 conidia/ml. The lunate conidia of *E. vermicola* adhered to the surface of PWN (Figure 6a,b). The germinated conidia penetrated the cuticle of *B. xylophilus*, and

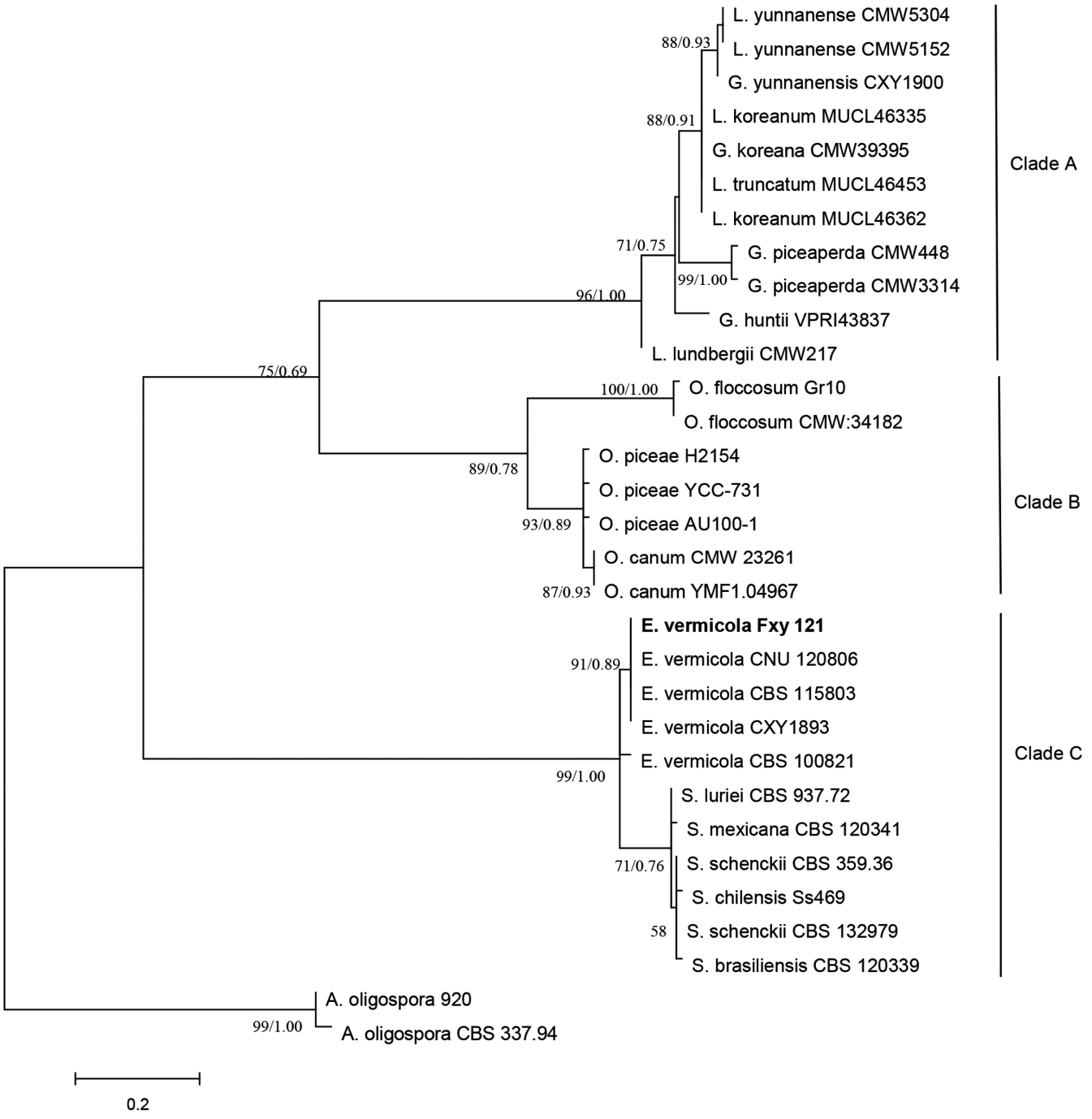

FIGURE 4 Phylogenetic analyses based on ML and BI analysis for *Esteya vermicola* Fxy121 from elongation factor-1α nucleotide fragment. Novel sequence obtained in this study is signed in bold type. Maximum likelihood bootstrap support values ((before the/) ≥50% were indicated with numbers. Bayesian posterior probability (BPP) support (after the/) ≥0.60 indicated at the branches. The tree is drawn to scale with branch length measured in the number of substitutions per site. Clade A–*Leptographium* spp. and *Grosmannia* spp., clade B—*Ophiostoma* spp., clade C—*Esteya* spp., and *Sporothrix* spp. The isolate Fxy121 grouped in clade C

hyphae colonized and grew in internal tissues (Figure 6c). Mycelia occupied the entire body of nematodes and produced many adhesive conidia, and the nematode was effectively digested to death (Figure 6d–f).

The adhesion of *E. vermicola* Fxy121 was 19.04% at 6 h after co-culture of *E. vermicola* and *B. xylophilus*, and increased to 77.77% at 24 h. After co-culture for 48 h, lunate conidia of *E. vermicola* adhered to 96.00% of *B. xylophilus* individuals. The mortality of PWN infected by *E. vermicola* was 57.80% at 48 h of co-culture and gradually increased to 74.5% at 96 h (Figure 7).

Thirty days after inoculation with PWN, the *P. massoniana* in Group PWN and EV showed clear symptoms (Figure 8). The symptom grades of all *P. massoniana* were shown in Table 3. Nine pine trees inoculation with PWN showed symptoms, while only 2 pine

trees showed symptoms after injection with *E. vermicola* and PWN. The infection of pine trees with PWN showed an infection rate of 90% and a DSI of 54%, while with *E. vermicola*, the disease severity index and infection rate were 20% and 10% respectively (Table 4). So the RCE of *E. vermicola* was about 81.48% after inoculation 30 days.

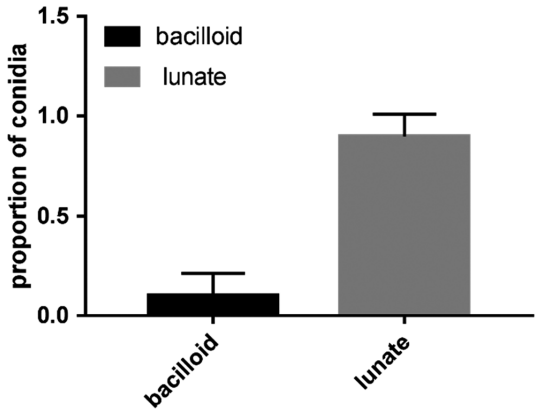

FIGURE 5 The proportion of conidia of *Esteya vermicola* cultures on PDA at 25°Cfor 10 days

DISCUSSION

In the present study, an isolate of *E. vermicola* was isolated from the bark of *P. yunnanensis* infested by *T. yunnanensis* in Panlong District, Kunming City, Yunnan, China. The β-tubulin sequence of our novel isolate, Fxy121, showed 100% similarity with the corresponding sequences of isolates NKF13222, CNU120806, and CBS 115803, and 99.75% similarity with the isolate Cxy1893, which differed in one nucleotide. In addition, Cxy1893 produced only lunate conidia in vitro (Wang et al., 2019), whereas Fxy121 produced lunate and bacilliform conidia, although the percentage of bacilliform conidia was only 10% on PDA medium (Wang et al., 2019). Although some differences between the two isolates were observed, we speculated that the Fxy121 and Cxy1893 isolates of *E. vermicola* might be the same geographic population based on their same hosts and location.

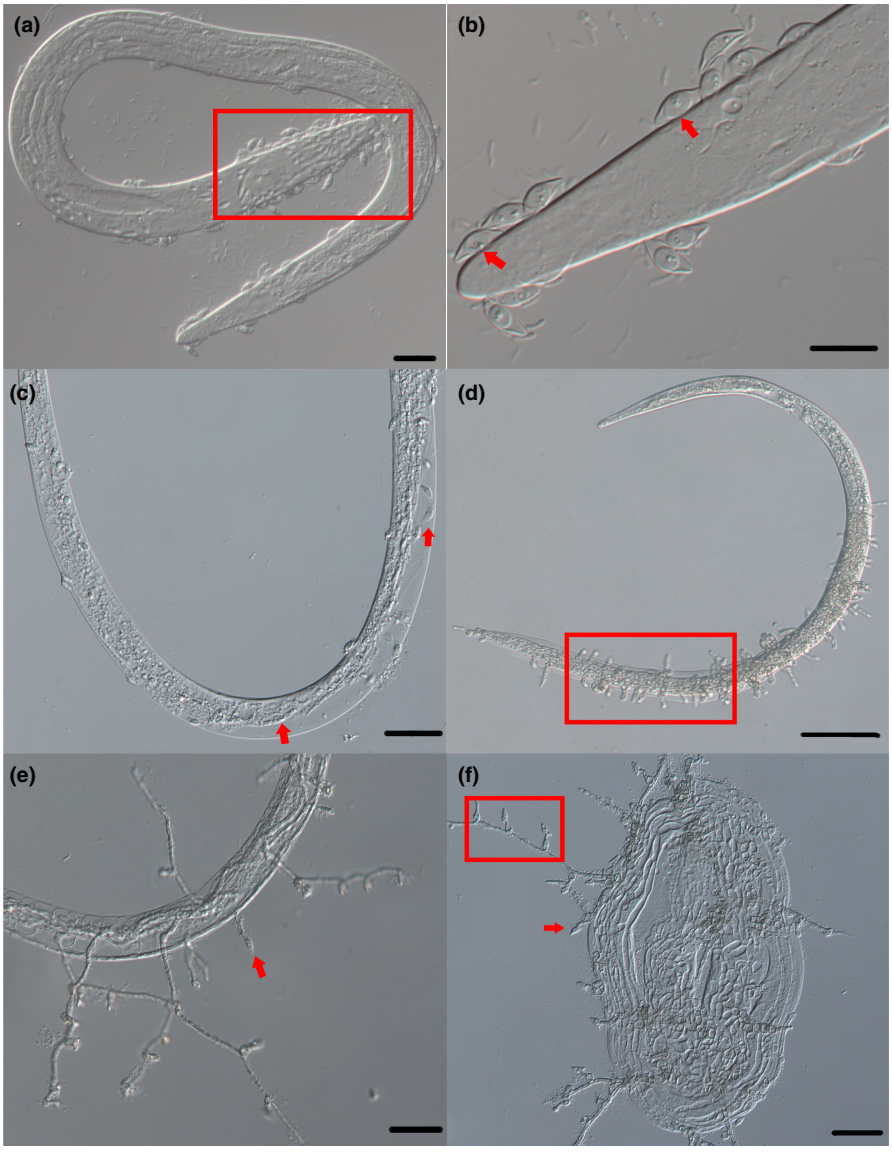

FIGURE 6 The morphology of *Esteya vermicola* and the infected nematode by it. (a) Lunate conidia adhered to the body of nematode; (b) infection peg initiated from a conidium penetrating the cuticle and muscle layer of a nematode (arrow heads); (c, d) the hyphae colonizing inner contents of nematode and grew hyphae; (e) the hyphae produced adhesive flask-shaped conidiogenous cells and lunate conidia; (f) nematode was destroyed and killed by the infection hyphae (lunate conidia from infection hyphae was shown with red box and arrow heads). Scale bars: 50 μm (a, d); 10 μm (b); 100 μm (c, e, f)

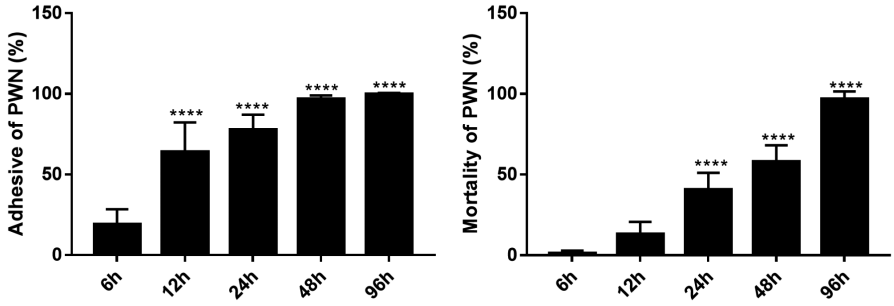

FIGURE 7 Adhesive rate and mortality of *Esteya vermicola*

FIGURE 8 Symptoms of *Pinus massoniana*. Trees in group W were inoculation with sterile water as negative control. Trees in group EV were inoculation with conidia suspensions of *Esteya vermicola* and suspensions of PWN as the treatment group. Trees in group PWN were inoculation with sterile water and suspensions of PWN as the positive control

Previous reports indicate that *E. vermicola* is distributed throughout the world, especially in Asia, Europe, and North America. Its hosts are also widely distributed, including nematodes (*B. xylophilus*, *B. rainulfi*, and saprophytic nematodes in soil), beetles (*S. intricatus*, *T. yunnanensis*, and *M. flavicornis*), and their host trees (*Olea europaea* L., *Quercus* spp., and *P. yunnanensis*). Our present novel isolate Fxy121 was obtained from pine trees, which are hosts of PWN (*B. xylophilus*). Thus, the novel isolate of *E. vermicola* shows potential as biological control agents effective against PWN based on their host origin.

Wang et al. (2008) observed that the CNU120806 isolate of *E. vermicola* isolated from infected nematodes in Korean forest soil showed high infection activity against nematodes on PDA medium. Almost all tested PWNs were killed within 8–10 days after inoculation. In 2016, Wang et al. (2016) compared variation in sporulation of four isolates of *E. vermicola* and their infectivity against PWN. The CBS 115803 isolate produced the highest proportion of infective lunate conidia, and accordingly exhibited the highest infectivity by killing all tested PWNs within 3 days. The amount and proportion of lunate infective conidia showed a positive correlation with infectivity against PWN. The novel isolate Fxy121 exhibited high adhesion and infection activity against PWN by adhering to all nematodes within 48 h and killing 74.5% of the tested PWNs within 4 days. The proportion of lunate infective conidia of Fxy121 was 90% when cultured on PDA medium. Thus, the high infection activity of this isolate might be closely associated with the high proportion of lunate conidia produced.

Up to now, there were few reports about the RCE of *Esteya* fungi against PWN in the field. Li et al. (2018, 2021) isolated a new species of the genus *Esteya* named *E. floridanum*. This fungus delayed the symptoms of pine wilt in greenhouse inoculation experiment (Li et al., 2021). Yin et al. (2020) have done the inoculation of PWN and *E. vermicola* CUN 120806. When injected with blastospores of *E. vermicola* 2 and 4 weeks after PWN infection, 40% of the trees were saved. In this research, 90% trees untreated with *E. vermicola* FXY121 and then inoculated with PWN turned yellow and 30% of them died within 4 weeks, while only 20% pine trees showed clear symptom after inoculation of PWN and *E. vermicola* FXY121. Our novel *E. vermicola* isolate delayed the symptoms of pine wilt in field and showed good potential as a bio-control agent against PWD in the field.

In summary, one isolate of *E. vermicola* was isolated from the bark of *P. yunnanensis* trunk in Kunming, Yunnan. This isolate showed a high proportion of infective lunate conidia and exhibited high infection activity against PWN. This isolate may be

TABLE 3 Symptom grade of inoculation *Pinus massoniana* in this study

Number	Symptom grade	Number	Symptom grade	Number	Symptom grade
W-1	0	EV-1	0	PWN-1	1
W-2	0	EV-2	0	PWN-2	0
W-3	0	EV-3	0	PWN-3	2
W-4	0	EV-4	0	PWN-4	5
W-5	0	EV-5	0	PWN-5	3
W-6	0	EV-6	0	PWN-6	3
W-7	0	EV-7	1	PWN-7	1
W-8	0	EV-8	4	PWN-8	5
W-9	0	EV-9	0	PWN-9	2
W-10	0	EV-10	0	PWN-10	5

W, inoculation with sterile water; **EV**, inoculation with conidia suspensions of *Esteya vermicola* and suspensions of *Bursaphelenchus xylophilus*; **PWN**, inoculation with sterile water and suspensions of *B. xylophilus*. 0, all needles were green; 1, 0%–20% of the needles turned yellow; 2, 20%–40% of the needles turned yellow; 3, 40%–60% of the needles turned yellow; 4, 60%–80% of the needles turned yellow; 5, 80%–100% of the needles turned yellow.

TABLE 4 Infection rates and disease severity index of *Pinus massoniana*

Treatment	Infection rates (%)	Disease severity index (DSI)
W	0	0
EV	20	10
PWN	90	54

well adapted to the parasitic environment within pine trees. This isolate also shows considerable promise as a bio-control agent against nematodes.

AUTHOR CONTRIBUTIONS

Conceptualization, Li YX and Zhang XY; methodology, Wang X and Wen XJ; software and formal analysis Zhang W and Li DZ; investigation, Wang X, Ze SZ and Liu ZK; resources, Wang X and Liu ZK; data curation, Wang X and Feng YQ; writing—original draft preparation, Wang X; writing—review and editing, Yong Xia Li and Xing Yao Zhang; supervision, Li YX and Zhang XY; project administration and funding acquisition, Li YX and Wang X. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Sequence data created and analysed in this research are openly available from Genbank® (https://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers for each data are available in the paper. Other data supporting the findings of this study are provided in full in the results section of this paper and available from the corresponding author upon request.

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SUPPORTING INFORMATION

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