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Identification and biocontrol potential evaluation of a naturally occurring *Metarhizium pingshaense* isolate infecting tea weevil *Myllocerinus aurolineatus* Voss (Coleoptera: Curculionidae)

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Abstract

Background Tea weevil, *Myllocerinus aurolineatus* Voss (Coleoptera: Curculionidae), is an important insect pest in Chinese tea plantations. The primary method for controlling tea weevils involves the use chemical pesticides. Hence, there is an urgent need for environmentally friendly control strategies. To screen for potential pathogenic strains useful for the biocontrol of tea weevils, a naturally occurring *Metarhizium pingshaense* strain was isolated from a field-collected infected tea weevil larva for the first time in China.

Results Morphological features and molecular characteristics revealed the isolate was an *M. pingshaense* strain, herein referred to as Ma0628. At 22 °C (tea weevil pupation temperature), the inoculation with *M. pingshaense* Ma0628 resulted in a corrected cumulative late instar larval mortality rate exceeding 76% at 11 days after the inoculation with the 1×10^8 conidia/ml spore suspension using the immersion or soil-mixing method. Accordingly, the median lethal concentrations were 4.49×10^3 and 3.76×10^2 conidia/ml for the immersion and soil-mixing inoculation methods, respectively. Furthermore, the corrected cumulative adult mortality rate reached 83.33% at 14 days after the inoculation with the 1×10^8 conidia/ml spore suspension.

Conclusion The study results indicate that *M. pingshaense* strain Ma0628 is an entomopathogenic fungus pathogenic to tea weevil larvae and adults, suggesting it may be a potentially useful biocontrol agent for preventing *M. aurolineatus* infestations.

Keywords Tea weevil, *Myllocerinus aurolineatus*, *Metarhizium pingshaense*, Identification, Pathogenicity, Biocontrol

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Background

Tea, which is made from the tender leaves of tea plants (*Camellia sinensis* O. Ktze), is one of the most consumed non-alcoholic beverages worldwide because of its health benefits, pleasant fragrances, and refreshing tastes (Fu et al. 2022). The tea weevil (*Myllocerinus aurolineatus* Voss; Coleoptera: Curculionidae) is a destructive leaf-feeding pest in Chinese tea plantations (Sun et al. 2012). The tea weevil has a 1-year life cycle, with adults typically emerging in early May and remaining until the end of June. Adult tea weevils infest the young leaves and shoots of tea plants, resulting in irregular arc-like notches on the tender leaves and leaving merely leaf veins when the pest outbreaks (Sun et al. 2017). The peak of *M. aurolineatus* infestation period coincides with the spring tea and summer tea harvest seasons in regions producing oolong tea and green tea, respectively. Consequently, the severe damages caused by tea weevils have substantially affected tea qualities and yields, leading to considerable economic losses to tea farmers (Sun et al. 2017). Unlike the adults, the tea weevil larvae live, active and overwinter in the soil with a depth of up to 30 cm right below the tea plant canopy from July to the following April. Our previous field investigations found that from mid-April onward, the larvae migrate to the 1–5-cm soil layer for pupation when the surface soil temperature reaches approximately 20 °C. Adequately controlling the larvae during this period can decrease the damages caused by tea weevil adults.

Tea weevils are currently controlled primarily via the application of chemical insecticides. However, using chemical insecticides may lead to an increased risk of pesticide residues in tea and tea products (Xu et al. 2022). In addition, some of these chemicals are potentially harmful to non-target beneficial organisms, with inevitable detrimental effects on the environment (Sutar et al. 2022). It is thus essential to develop environmentally friendly strategies to control this tea pest.

Biocontrol agents are crucial alternatives to pesticides and have become important components of integrated pest management practices contributing to modern sustainable agriculture. Entomopathogenic fungi (EPF), such as strains belonging to the genera *Metarhizium* and *Beauveria*, are representative microbial biocontrol agents effective against insect pests, with little or no adverse effects on the environment (Bamisile et al. 2021). These fungi can degrade the cuticle of insect pests, proliferate in the hemolymph as hyphal bodies, secrete toxins that can kill the host pests, and produce spores capable of re-infecting other host pests (Sharma et al. 2023). Apart from being insect pathogens, EPF could be also used as endophytes, plant growth promoters, rhizosphere colonizers, disease antagonists, etc. (Bamisile et al. 2021). These multiple ecological roles played by EPF make them ideal candidates for use in sustainable agriculture. To date, 171 EPF strains have been formulated as mycopesticides, which are commercially available worldwide for controlling pests (Kumar et al. 2019). More specifically, approximately 34% of these mycopesticides consist of species from the genus *Metarhizium*, which is a rapidly expanding lineage comprising more than 60 species (Senthil et al. 2023).

To develop potential EPF-based strategies for tea weevil biocontrol, in the present study, one native EPF isolate was obtained from a naturally infected tea weevil larva, which was collected from an organic tea plantation where *M. aurolineatus* outbreak occurs. The isolate was identified as *Metarhizium pingshaense* based on morphological characterization and multi-gene sequence analysis. Moreover, the pathogenicity of the isolated *M. pingshaense* strain (Ma0628) to *M. aurolineatus* larvae and adults was assessed under laboratory conditions. The results reflected the potential utility of Ma0628 as a biocontrol agent effective against *M. aurolineatus*. This strain may be useful for developing novel biocontrol methods to limit the damages caused by tea weevils.

Methods

Specimen collection and fungal isolation

A naturally infected tea weevil larva wholly covered with fungal mycelia and adhering to soil was collected from the Pingyang organic tea garden in Pingshui county, Shaoxing city, Zhejiang province, China (120.61122° E, 29.84760 °N) on April 12, 2021. To isolate the fungus, the cadaver of the infected tea weevil larva was surface-sterilized in 75% ethanol for 5 min on a clean bench and then rinsed three times with sterile distilled water to remove any adhering soil. The cadaver was cut into small pieces using sterile surgical scissors. The cut pieces were used to inoculate potato dextrose agar (PDA) medium plates amended with 0.1 g/l chloramphenicol, with three pieces per plate (added to form an equilateral triangle). The plates were incubated at 27 ± 1 °C for 7 days in a biochemical oxygen demand (BOD) incubator. The fungi were purified by sub-culturing to obtain pure colonies. The PDA plates containing pure colonies were stored at 4 °C for the subsequent analysis.

Morphological identification

Spores of the isolated and purified colonies were collected using sterile pipette tips for the inoculation of PDA medium plates amended with 0.1 g/l chloramphenicol, which were then incubated at 27 ± 1 °C in a BOD incubator. For the mycelium, conidiophore structure, and spore observation, the spores were streaked across a PDA plate in a zig-zag motion. Sterilized coverslips were inserted at 45° angles next to the inoculation lines. To examine the

morphological characteristics of the mycelium, conidiophore structures, and spores, the sterile coverslips were analyzed using the Keyence VHX-7000 microscope (Keyence, Shanghai, China) at 5–10 days post-inoculation (DPI). For colony feature examination, 100 µl 0.05% (v/v) Tween-80 solution containing spores (20–30 conidia/ml) was spread evenly over the surface of PDA plates using a sterile bent glass rod. The colony appearance and pigmentation on the underside of the PDA plate were recorded at 10 DPI.

DNA extraction, PCR amplification and sequencing

The mycelium and conidia of the isolated strain were collected from the PDA medium at 5 DPI, after which genomic DNA was extracted using the Ezup Column Fungi Genomic DNA Purification kit (Sangon Biotech, Shanghai, China). The translation elongation factor 1α gene (*EF-1α*) and β-tubulin gene (*TUB*) were amplified by PCR. Specifically, the 5′ terminal sequence of *EF-1α* (approximately 1200 bp) was amplified using primers EF1T and 1567R, whereas the 3′ terminal sequence was amplified using primers tef1fw and 1750-R (López et al. 2022; Senthil et al. 2023). The Pbeta-F and Pbeta-R primers were used to amplify the *TUB* gene (Wei et al. 2023). The PCR amplifications were carried out using 50-µl solutions containing 30–60 ng genomic DNA, 25 µl 2×Taq Master Mix (Vazyme, Nanjing, China), 1 µl forward and reverse primers (10 µmol/l each), and PCR-grade water. Information regarding the PCR primers is provided in Table 1. The PCR conditions were as follows: 95 °C for 3 min; 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 80 s; and the final elongation was at 72 °C for 5 min. The PCR products were inserted into the pEASY®-Blunt Zero vector (Transgen, Beijing, China), and positive colonies were selected for sequencing.

Sequence alignment and phylogenetic analysis

The sequencing results for the 5′ and 3′ terminal sequences of *EF-1α* and *TUB* were manually trimmed using the Editseq software. The full-length *EF-1α* sequence was obtained by assembling the 5′ and 3′ terminal sequences using the SeqMan software. Homologs of the *EF-1α* and *TUB* sequences were identified via a Basic Local Alignment Search Tool (BLAST) search. Highly similar reference sequences from species in the genus *Metarhizium* were downloaded. The sequences from the isolated fungus and closely related species were aligned, using the ClustalW multiple sequence alignment tool in the MEGA 5.0 software. Phylogenetic trees were constructed using the neighbor-joining method and MEGA 5.0, with 1,000 bootstrap replicates. *Beauveria bassiana* strains ARSEF 7257 (accession number: AY883707.1) and ARSEF 2860 (accession number: XM008602115.1) were used as the out-group for *EF-1α* and *TUB*, respectively.

Table 1 Primers used for amplifying *EF-1α* and *TUB* sequences

Name	Sequence
EF1T	ATGGGTAAGGARGACAAGAC
1567R	ACHGTRCCRATACCACCSATCTT
tef1fw	GTGAGCGTGGTATCACCA
1750-R	GACGCATGTCACGGACGG
Pbeta-F	ACCCTCCATTGTCTAGGACC
Pbeta-R	CACATCATTGACGGGACTTAC

Preparation of spore suspensions

To collect spores, the isolated strain was used to inoculate PDA medium overlaid with a transparent cellophane membrane (Sangon Biotech, Shanghai, China). After a 14-day incubation at 27 ± 1 °C, the spores were washed with sterilized 0.05% Tween-80 solution. The spore solution was mixed with a magnetic bead for 30 min to thoroughly disperse the spores, after which the conidia were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA) and the Keyence VHX-7000 microscope (Keyence). The spore suspension was diluted for spore concentrations of 1.0×10^8, 1.0×10^7, 1.0×10^6, and 1.0×10^5 conidia/ml for the subsequent experiments.

Bioassays to determine the pathogenicity of *M. pingshaense* strain Ma0628 to tea weevils *M. aurolineatus*

Bioassay of the larvae

Mature *M. aurolineatus* larvae were collected from the Pingyang organic tea garden in March and April 2022–2023. The larvae were reared in soil collected from the same organic tea garden in an artificial climate chamber set at 22 ± 1 °C, with a 16-h light:8-h dark cycle and 70% ± 5% relative humidity (RH). Because the tea weevil larval instars have not been characterized, healthy larvae that were approximately the same size were selected for the pathogenicity bioassay. The immersion and soil-mixing methods were used for the spore inoculation. For the immersion method, the larvae were immersed in 2-ml spore suspensions (with different concentrations) for 20 s and then placed on filter paper to absorb the excess liquid. The larvae were transferred to a rearing cup (height and width: 4 cm × 4.5 cm) containing 20 g soil (22% RH). Larvae treated with a sterilized 0.05% Tween-80 solution reared under the same conditions served as the control group for the immersion method. For the soil-mixing method, 20 g soil (22% RH) was thoroughly mixed with a 2-ml spore suspension (with different concentrations) and then added to the rearing cup. Healthy larvae were subsequently transferred to the rearing cup. Larvae

reared in soil treated with a sterilized 0.05% Tween-80 solution were used as the control group for the soil-mixing method. For each spore concentration, three groups of larvae were analyzed, with each group comprising 10 larvae. All spore-treated and control larvae were incubated in an artificial climate chamber (22 ± 1 °C, 70% ± 5% RH, and 16-h light:8-h dark cycle). Starting on day 3 of the incubation, the larvae were examined for 8 consecutive days and the larval mortality rate of each group was recorded daily. The dead larvae were surface-sterilized, transferred to sterilized Petri dishes lined with moistened filter paper, and incubated in the same condition as above.

Bioassay of the adults

The virulence of *M. pingshaense* strain Ma0628 to adult tea weevils was assessed with the following procedures. Briefly, tea weevil adults were collected from the organic tea garden and reared on fresh tea shoots in an artificial climate chamber (25 ± 1 °C, 70% ± 5% RH, and 16-h light:8-h dark cycle). The adults were inoculated with a spore suspension (1.0×10^8 conidia/ml) using the immersion method. The control group consisted of adults treated with a sterilized 0.05% Tween-80 solution. The spore-inoculated and control adults were reared on fresh tea shoots in insect-rearing cages (length, width, and height: 20 cm × 18 cm × 38 cm). The cages were maintained at room temperature (24–28 °C), with a 16-h light:8-h dark cycle and 70 ± 5% RH. Three groups (10 adults each) were analyzed for the spore-inoculated and control samples. The adults were examined for 14 consecutive days, and the adult mortality rate of each group was recorded daily. The dead adults underwent the same treatment as the dead larvae.

Statistical analysis

Experimental data were recorded and processed using Excel. The mortality rate and corrected mortality rate were calculated using the following equations:

$$\text{mortality rate (\%)} = \frac{\text{number of dead in sects}}{\text{number of experimental insects}} \times 100$$

$$\text{corrected mortality rate (\%)} = \frac{(\text{treated mortality rate} - \text{control mortality rate})}{(100 - \text{control mortality rate})} \times 100$$

The larval mortality rate was corrected using Abbott's formula (provided above) because the control mortality rate was ≥ 5% and ≤ 20%. The adult mortality rate did not need to be corrected because the control mortality rate was < 5%. The median lethal concentration (LC_{50}) values were calculated according to the Probit method in SPSS 20. The corrected larval mortality rates for the different spore concentrations were subjected to a one-way analysis of variance (ANOVA) with a post hoc Tukey's test using GraphPad Prism® (version 5.0) (GraphPad Software, La Jolla, California, USA). Student's *t*-test was used to analyze the corrected larval mortality rates for the different inoculation methods. It was also used to compare the adult mortality rates for the spore-inoculated and control groups. The threshold for determining significant differences between groups was $P < 0.05$. All graphs presented herein were plotted using GraphPad Prism® (version 5.0).

Results

Morphological identification

Colonies of the isolated fungus (Ma0628) grown on PDA medium were initially ivory white and the aerial hyphae were slight, with a thin, white, downy, fluffy, or floccose mat forming at the distal parts of the colony (Fig. 1a). Upon sporulation, the colonies appeared grayish, with a light yellowish gray underside. Green conidia gradually started to appear in the middle of the colonies (Fig. 1b–c). Under a light microscope, the hyphae were smooth, colorless, separated, and branched (width approximately 1.6–2.8 µm) (Fig. 1d). The conidiophores, which arose singly or in loose groups, were erect or somewhat flexuous and slightly swollen at the tip, with branched meristematic apices that produced single-celled conidia in basipetal succession (Fig. 1e–g). The single-celled conidia (2.39–3.66 × 5.73–7.67 µm) were transparent with a smooth surface and columnar or ellipsoidal with tapered ends (Fig. 1g). Individual conidium was adhering laterally forming long chains (Fig. 1h).

Molecular characterization

The PCR amplification of the 5′ and 3′ termini of the *EF-1α* gene and the *TUB* gene for the isolated strain (Ma0628) yielded amplicons of the size 1228 bp, 1154 bp, and 1316 bp, respectively. After combining the 5′ and 3′ terminal fragments, the full-length *EF-1α* sequence consisted of 1734 bp. The BLAST search for *EF-1α* homologs revealed the similarity between Ma0628 and *M. pingshaense* type strains. Specifically, the Ma0628 *EF-1α* sequence was 99.94% similar to sequences in *M. pingshaense* CBS257.90 (EU248850.1) and ARSEF 7929 (EU248847.1). Moreover, the Ma0628 *EF-1α* sequence shared more than 99.85% similarity to the corresponding sequences in another 11 M.

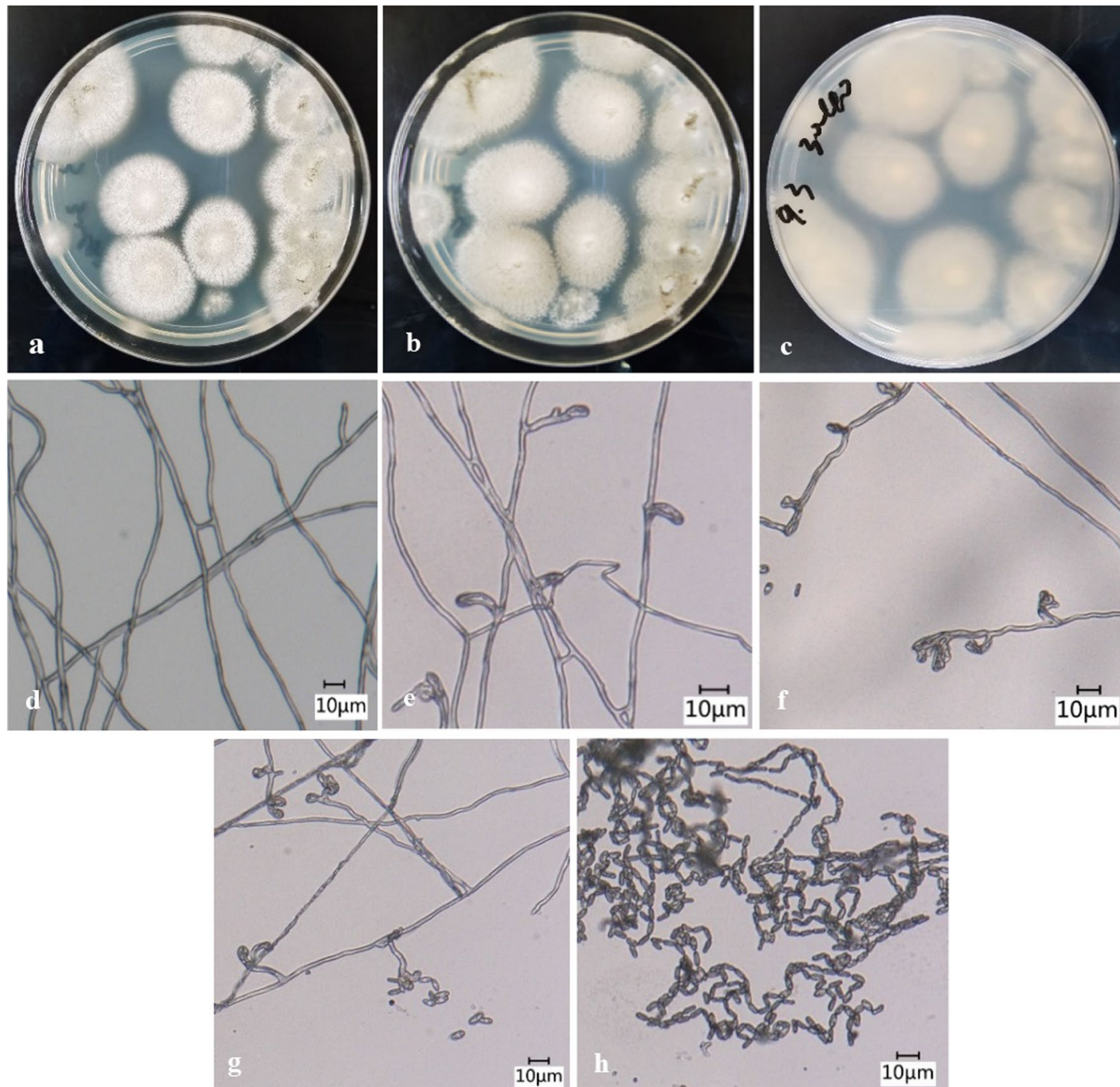

Fig. 1 Morphological characteristics of *M. pingshaense* strain Ma0628. **a**–**b** Colonies on the medium surface; **c** underside of the plate; **d** mycelium; **e**–**g** conidiophore structure; **h** spores

pingshaense strains. In the phylogenetic tree constructed for *EF-1α* sequences, Ma0628 was clustered in a branch containing only *M. pingshaense* strain with 99% bootstrap support (Fig. 2). The Ma0628 *TUB* sequence was identical to the homologous sequences in *M. pingshaense* strains ARSEF 7929 (EU248847.1) and ARSEF 3210 (EU248819.1). The phylogenetic tree constructed for *TUB* sequences revealed that Ma0628 was grouped with *M. pingshaense* on the same branch (98% bootstrap support) (Fig. 3). In conclusion, the phylogenetic analysis of *EF-1α* and *TUB* sequences combined with the observed morphological features identified Ma0628 as *M. pingshaense*.

Pathogenicity of *M. pingshaense* Ma0628 to *M. aurolineatus*

The pathogenicity of Ma0628 to the tea weevil at 22 °C (pupation temperature) was analyzed by evaluating the susceptibility of the larvae using the immersion and soil-mixing methods. Four different conidial concentrations (1×10^5–1×10^8 conidia/ml) were tested. Regardless of the inoculation method and spore concentration, the cumulative mortality rate of the tea weevils increased as the duration of the treatment period increased (Fig. 4). For the immersion method, the cumulative mortality rates of the larvae inoculated with 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml were 60.00%, 63.33%, 73.33%, and 76.67%, respectively, on day 11 (Fig. 4a and Additional file 1: Table S1).

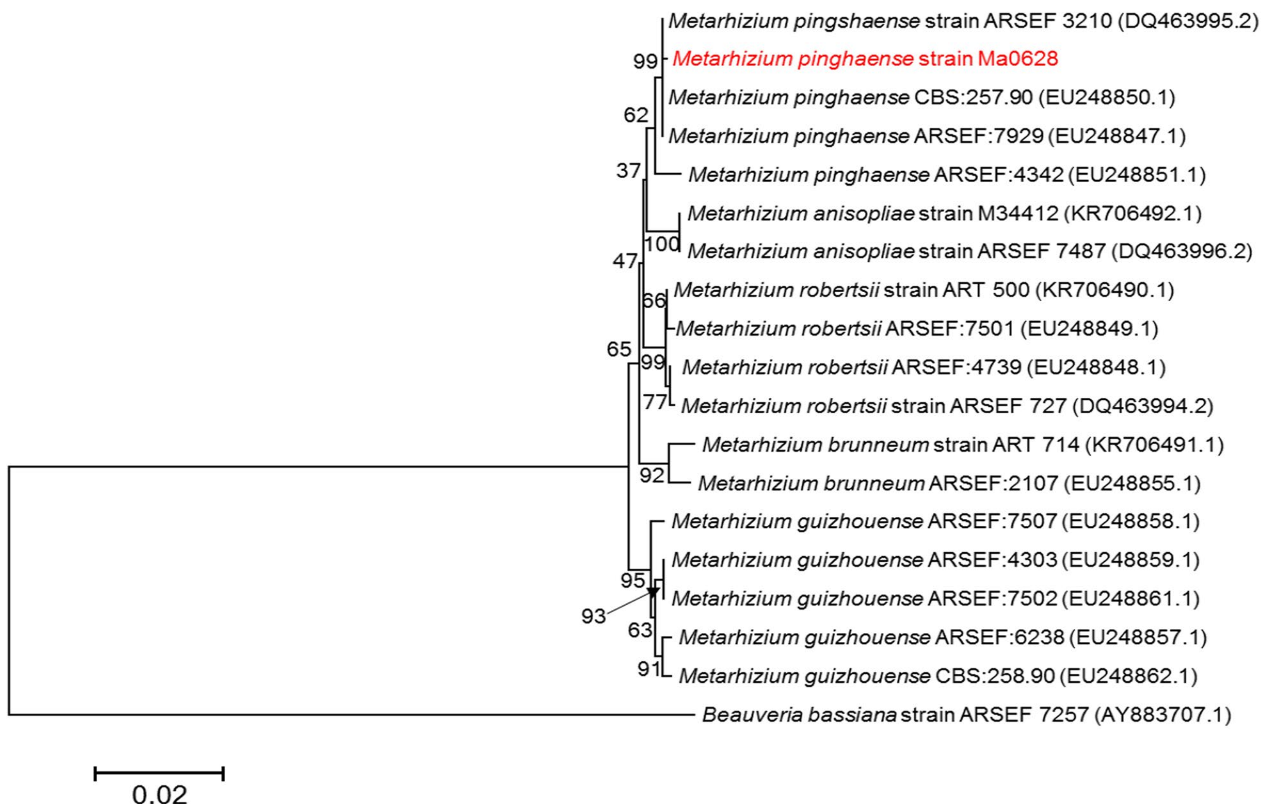

Fig. 2 Phylogenetic tree comprising *M. pingshaense* strain Ma0628 and closely related strains. The tree was constructed using *EF-1α* sequences with the neighbor-joining method. The numbers in parentheses represent GenBank accession numbers. Bootstrap support values are provided at each branch point. The scale bar for the branch represents 0.02

For the soil-mixing method, the cumulative mortality rates on day 11 were 60.00% and 76.67% for the larvae inoculated with 1×10^5 and 1×10^8 conidia/ml, respectively. The cumulative mortality rates for the larvae inoculated with 1×10^6 and 1×10^7 conidia/ml were 76.67% and 80.00%, respectively, on days 10 and 11 (Fig. 4b and Additional file 1: Table S1). The corrected larval mortality rate at 11 DPI is provided in Table 2. The inoculation with different concentrations of *M. pingshaense* Ma0628 spores resulted in a corrected larval mortality rate exceeding 59.80%. For the soil-mixing method, the corrected larval mortality rates were 79.8% and 76.47%, when the spore concentrations were 1×10^7 and 1×10^8 conidia/ml, respectively; this difference was not significant. Moreover, there were no significant differences between the two inoculation methods according to Student's *t*-test or among the spore concentrations according to the one-way ANOVA with a post hoc Tukey's test (Table 2).

The LC_{50} values for the tea weevil larvae inoculated with *M. pingshaense* Ma0628 spore suspensions are listed in Table 3. Because the mortality rate exceeded 50% for both inoculation methods only from 6 DPI, LC_{50} was calculated from day 6 to day 11. On day 6, the LC_{50} values were 8.92×10^7 and 3.00×10^7 conidia/ml for the immersion and soil-mixing methods, respectively (Table 3). For both inoculation methods, the LC_{50} gradually decreased during the post-inoculation period. With the exception of day 9, on each day after the spore inoculation, LC_{50} was slightly lower for the soil-mixing group than for the immersion group, although the differences were not significant.

The pathogenicity of Ma0628 to the tea weevil adults was also evaluated by analyzing the susceptibility of the adults inoculated with the spore suspension comprising 1×10^8 conidia/ml using the immersion method. Dead tea weevil adults were detected starting from 3 DPI (Fig. 5 and Additional file 1: Table S2). Additionally, the adult mortality rate gradually increased over time, reaching 83.33% at 14 DPI. In contrast, the adult mortality rate was only 3.33% in the control group immersed in 0.05% Tween-80.

There were non-significant differences between the two inoculation methods (Student's *t*-test) or among the spore concentrations (one-way ANOVA with a post hoc Tukey's test).

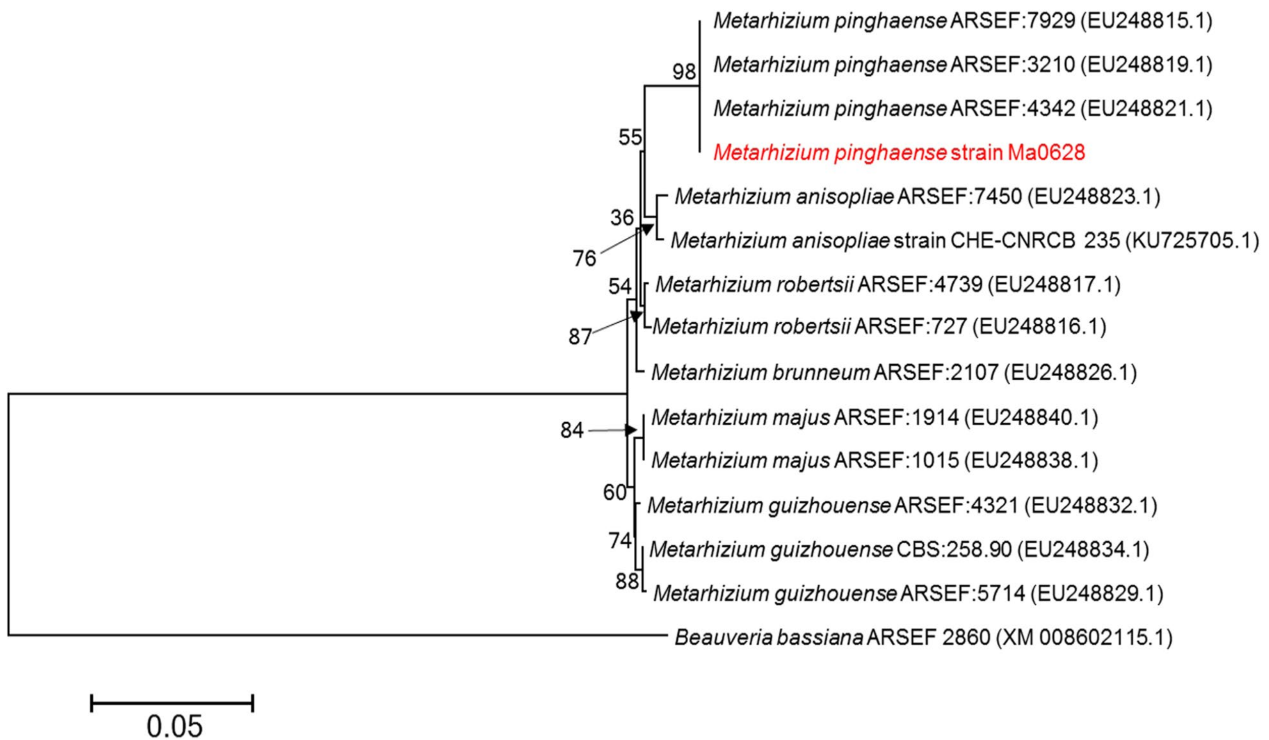

Fig. 3 Phylogenetic tree consisting of *M. pingshaense* strain Ma0628 and closely related strains. The tree was constructed using *TUB* sequences with the neighbor-joining method. The numbers in parentheses represent GenBank accession numbers. Bootstrap support values are provided at each branch point. The scale bar for the branch represents 0.05

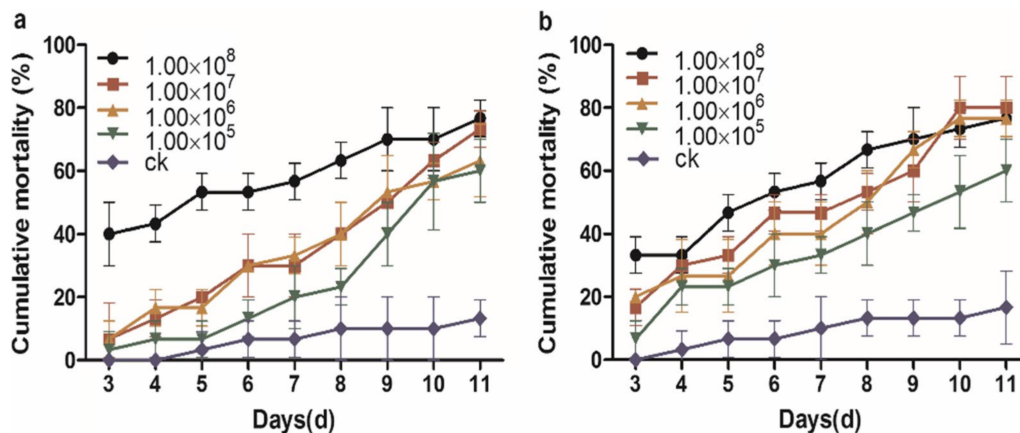

Fig. 4 Pathogenicity of *M. pingshaense* strain Ma0628 to *M. aurolineatus* larvae. **a** Larvae inoculated with immersion method; **b** Larvae inoculated with soil-mixing method; 1×10^{5}–1×10^{8} refer to different *M. pingshaense* strain Ma0628 spore concentrations; ck represents the control group treated with 0.05% Tween-80. Data are presented as the mean ± standard deviation. For additional details, see Additional file 1: Table S1

LC_{50} represents the concentration required to kill 50% of the insects in the tested population.

The larvae and adults that died following the inoculation with *M. pingshaense* Ma0628 were covered with white mycelia and olive-green spores (Fig. 6b–d). In addition, clay bumps were found on the surface of the infected larvae, but not on the surface of the uninfected larvae (Fig. 6a–b). And no mycelia and spores were observed on the dead larvae and adults in control group. Spores were isolated from the dead tea weevil larvae and adults infected with *M. pingshaense* Ma0628 according to Koch's postulates. Both the morphological features and *EF-1α* and *TUB* sequences were the same as those of *M. pingshaense* Ma0628 (data not shown), indicating the

Table 2 Corrected mortality rates for *M. aurolineatus* larvae at 11 days post-inoculation

Spore concentrations (spores/ml)	Corrected mortality rate of larvae inoculated with different methods (%)	
	Immersion	Soil mixing
10^8	76.51	76.47
10^7	73.18	79.80
10^6	63.18	76.47
10^5	59.85	59.80

dead tea weevil larvae and adults died indeed from *M. pinghaense* Ma0628 infection. Therefore, *M. pingshaense* Ma0628 is a fungal pathogen of tea weevils, making it a potential biocontrol agent for preventing tea weevil infestations.

Discussion

Entomopathogenic fungi are important natural enemies of various insect pests, suggestive of their potential utility as biocontrol agents that can improve the production of horticultural and agricultural plants. Earlier research confirmed EPF from the genus *Metarhizium* is pathogenic to more than 200 insect species in seven orders, and some of these EPF having been developed as commercial biocontrol agents (Patel 2022). Previous studies on *M. pingshaense* isolates had demonstrated their pathogenicity and biocontrol potential to the red palm weevil (*Rhynchophorus ferrugineus*), subterranean termite (*Odontotermes obesus*), mosquito (*Anopheles coluzzii*), rice leaf folder (*Cnaphalocrocis medinalis*), and yellow peach moth (*Conogethes punctiferalis*) (Senthil et al. 2021). In the present study, the infection of a tea leaf-chewing pest, tea weevil (*M. aurolineatus*), was detected for the first time by the naturally occurring pathogenic fungus *M. pingshaense* Ma0628. The possibility that Ma0628 may be useful for the biocontrol of tea weevils was evaluated under laboratory conditions. The findings of this study provide the basis for developing *M. pingshaense*-based biocontrol strategies for managing tea weevil infestations in Chinese tea plantations.

Traditionally, macro- and micro-morphological features, including colony characteristics, color, shape, and size of the conidiophores and conidia, are used to delineate novel EPF species (Mayerhofer et al. 2019). As the number of new species in the genus *Metarhizium* continues to increase, researchers have found the overlapping morphological features of the conidia produced by the species in the *M. anisopliae* complex, making it difficult to distinguish between these species based on morphological traits alone with few exceptions (Mayerhofer et al. 2019). Indeed, the morphological characteristics

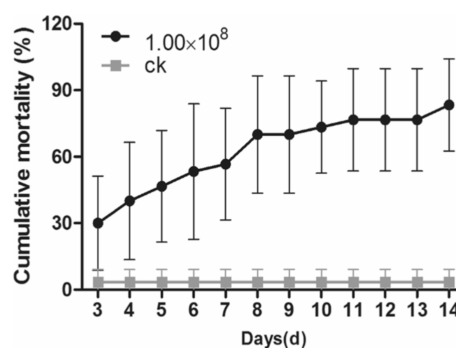

Fig. 5 Pathogenicity of *M. pingshaense* strain Ma0628 to *M. aurolineatus* adults. 1×10^8 refers to the Ma0628 spore concentration; ck represents the control group treated with 0.05% Tween-80. Data are presented as the mean ± standard deviation. For additional details, see Additional file 1: Table S2

Table 3 Regression equation for the pathogenicity of *M. pingshaense* strain Ma0628 to *M. aurolineatus* larvae

Days after inoculation	Inoculation methods	Probit regression equation	LC_{50} (conidia/ml)
6	Immersion	$y = -2.821 + 0.355x$	8.92×10^7
	Soil mixing	$y = -1.485 + 0.694x$	3.00×10^7
7	Immersion	$y = -2.334 + 0.297x$	7.38×10^7
	Soil mixing	$y = -1.429 + 0.197x$	1.83×10^7
8	Immersion	$y = -2.297 + 0.319x$	1.59×10^7
	Soil mixing	$y = -1.318 + 0.213x$	1.57×10^6
9	Immersion	$y = -0.502 + 0.123x$	1.17×10^4
	Soil mixing	$y = -0.800 + 0.166x$	6.57×10^4
10	Immersion	$y = -502 + 0.123x$	1.17×10^4
	Soil mixing	$y = -0.628 + 0.183x$	2.75×10^3
11	Immersion	$y = -0.623 + 0.171x$	4.49×10^3
	soil mixing	$y = -0.415 + 0.161x$	3.76×10^2

Fig. 6 Morphology of tea weevil larvae and adult. **a** Uninfected larva; **b** infected larva; **c** dorsal view of an infected adult; **d** ventral view of an infected adult

of Ma0628 strain were basically consistent with those of *Metarhizium anisopliae* and *M. pingshaense* (Francis and Manchegowda 2023; Zhao et al. 2023). Bischoff et al. (2009) have successfully established a reliable multigene phylogenetic approach, which used near-complete sequences from nuclear encoded *EF-1α*, *RPB1*, *RPB2*, and *TUB* gene regions to differentiate species within the *M. anisopliae* complex. To confirm the taxonomic status and morphological identity of *M. pingshaense* Ma0628, we amplified and sequenced the nearly full-length *EF-1α* and *TUB* sequences to search for homologs and conducted a phylogenetic analysis. Both *EF-1α* and *TUB* from target-isolated fungus were highly similar to sequences in the authentic *M. pingshaense* reference strains reported earlier. The phylogenetic trees constructed for the *EF-1α* and *TUB* genes included the corresponding reference taxa for Metarhizium species and placed Ma0628 in the *M. anisopliae* clade along with *M. pingshaense* reference strains, confirming Ma0628 as an *M. pingshaense* strain.

Bioassays showed that the *M. aurolineatus* larval mortality rate was affected by the *M. pingshaense* Ma0628 spore concentration. Following the inoculation with 1×10^8 conidia/ml, the corrected cumulative mortality rate was higher than 76% at 11 DPI for the *M. aurolineatus* both late instar larvae and adults, reflecting the virulence of Ma0628. Unlike the larvae, tea weevil adults have a rigid protective exoskeleton and elytra, which may provide protection against an infection by *M. pingshaense* Ma0628. For the immersion method, Ma0628 (1×10^8 conidia/ml) was equally pathogenic to the larvae and adults at 11 DPI, possibly because different incubation temperatures were used for the larva and adult bioassays. To mimic field conditions, the larvae were maintained at 22 °C (pupation temperature), whereas the adults were reared at room temperature (24–28 °C), which was optimal for the growth of EPF (Masoudi et al. 2018). It was previously determined that tea weevil larvae migrate to the 1–5-cm soil layer for pupation when the surface soil temperature is approximately 20 °C in April. To control the tea weevils during this period, the pathogenicity of Ma0628 to late instar larvae was assessed using the immersion and soil-mixing methods. Except for day 9, the LC_{50} values were slightly lower (but non-significant) in the soil-mixing group than in the immersion group after the spore inoculation. It was speculated that the upward migration of the larvae increased the likelihood of infection for the larvae treated using the soil-mixing method. Specifically, for the soil-mixing method, the LC_{50} values for the late instar larvae were 1.83×10^7 and 3.76×10^2 conidia/ml at 7 and 11 DPI, respectively. In earlier studies involving *M. pingshaense*, the LC_{50} values were 9.6×10^4 and 9.1×10^5 conidia/ml for the larvae of the moth species *Ectropis obliqua* and *C. punctiferalis*, respectively (Zhao et al. 2023). However, comparing the results of different studies is complicated by the diversity in the fungal isolates, insect pests, inoculation methods, and incubation temperatures.

To make microorganisms applicable as a biological control agent, isolation of native isolates present in the pest's natural environment is an important first step (Mann and Davis 2021). In this study, *M. pinghaense* Ma0628 isolate was obtained from naturally infected tea weevil larvae in an organic tea plantation where the tea pest outbreaks. This is important for the potential utility of this strain because tea plants reportedly grow in aluminum-enriched acidic soils, with an extremely acidic mean pH of 3.86 (Huang et al. 2023). Therefore, *M. pingshaense* Ma0628 may have adapted to the soil conditions in the tea garden. Our laboratory bioassay showed *M. pingshaense* Ma0628 exhibited promising pathogenicity to tea weevil larvae and adults. In addition, profuse sporulation was observed on both larval and adult tea weevil cadavers. The spores can help disperse the fungus under field conditions, resulting in epizootics (Shan and Feng 2010). These results suggested that Ma0628 can be developed as a potential biocontrol agent for restricting tea weevil infestations.

However, the toxicity of Ma0628 to tea weevils under laboratory conditions may not be indicative of the toxicity under field conditions, because environmental factors, including temperature, humidity, and ultraviolet (UV) radiation, are far more complicate and variable in the field than in a laboratory with controlled conditions. Numerous studies have proven that the abiotic factors have unneglectable impacts on the viability and biocontrol efficacy of *Metarhizium* species. For instance, exposure to high levels of UV radiation, lesser RH, and unfavorable temperatures (< 20 °C and > 35 °C) can decrease the virulence of EPF (Cheong and Azmi 2020; Couceiro et al. 2021; Kamga et al. 2022; Rossouw et al. 2023) Therefore, to develop *M. pingshaense* Ma0628-based mycoinsecticides for controlling tea weevils in tea plantations, further explorations on the stability and virulence in the field condition are urgently needed.

Conclusion

In this study, the naturally occurring *M. pingshaense* Ma0628 strain was isolated from a field-collected infected tea weevil larva for the first time in China. The laboratory bioassays results indicated that the strain is an entomopathogenic fungal with excellent pathogenicity to tea weevil larvae and adults. Therefore, the *M. pingshaense* Ma0628 strain could be used as a candidate biocontrol strain for managing *M. aurolineatus* in tea plantations. To develop *M. pingshaense* Ma0628 as a mycoinsecticide effective against tea weevils, the stability and virulence of the strain in tea gardens need to be assessed.

Abbreviations

EPF	Entomopathogenic fungi
PDA	Potato dextrose agar
BOD	Biochemical oxygen demand
DPI	Days post-inoculation
EF-1α	Translation elongation factor 1α
TUB	β-Tubulin
BLAST	Basic local alignment search tool
RH	Relative humidity
LC_{50}	Median lethal concentration
ANOVA	One-way analysis of variance
UV	Ultraviolet

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-023-00749-1.

Additional file 1: supplementary Table S1.

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Author contributions
NF conceived the research, conducted the experiments, analyzed the data, and wrote the manuscript. TW performed the PCR-based cloning and sequencing, analyzed the sequences, and edited the manuscript. QL, ZL, ZL, LB, CX, and ZC helped interpret the data and review and edit the manuscript. XC helped design the experiments, interpret the data, and review and edit the manuscript.

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Availability of data and materials
All datasets for this study are provided either in the manuscript or in the Additional file 1: Table S1.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests.

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