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ENVIRONMENTAL BIOTECHNOLOGY

Elevational distribution and morphological attributes of the entomopathogenic fungi from forests of the Qinling Mountains in China

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Abstract

Entomopathogenic fungi are considered to be a safe microbiological pesticide alternative to chemical control. Efforts are underway to understand precisely their taxonomy and natural distribution through mycological and biodiversity studies based on molecular markers. Here, we present descriptions of the diversity of the entomopathogenic fungi in the genera *Metarhizium* and *Beauveria* found along the elevational gradients of the Qinling subtropical and temperate forests of Shaanxi province in China, using morphological aspects and molecular markers. Molecular characterization using the *Mz_IGS3* intergenic region revealed that *Metarhizium* isolates phylogenetically clustered in the PARB clade with four different distinguishable species, but the 5′-*TEF* gene allowed only ambiguous delimitation of *Metarhizium* species. *Beauveria* isolates were characterized by sequence analyses of the translation elongation factor 1-α and the *Bloc* region. The richness of *Metarhizium* species decreased with increasing elevation, with *Metarhizium robertsii* s.l. being the most abundant species along the elevational gradient. Our bioassay suggests that certain species of *Metarhizium* are significantly pathogenic to the insect model *Tenebrio molitor* at both the adult and larvae stages and could potentially serve as a control of insect pests of forests.

Keywords Microbial ecology · *Metarhizium* · *Beauveria* · Pathogenicity · Molecular taxonomy · Microbial control

Introduction

Phylogenetic analysis has shown that two genera of insect pathogenic fungi (referred to as IPF below), *Metarhizium* (*Hypocreales*, *Clavicipitaceae*) and *Beauveria* (*Hypocreales*, *Cordycipitaceae*) (teleomorph *Cordyceps*), may have evolved from fungi that were plant associates (Gao et al. 2011; Spatafora et al. 2007). But, there is little evidence that the natural distribution of *Metarhizium* and *Beauveria* species in natural habitats is associated with plant types (Bidochka et al. 2001, 2005; Kepler et al. 2015; Keyser et al. 2015; Meyling and Eilenberg 2007; Wyrebek et al. 2011). The relative occurrence of *Metarhizium* with *Beauveria* differs across forest environments (Popowska-Nowak et al. 2016; Vanninen 1996). *Metarhizium* and *Beauveria*, the green and white muscardine anamorphic entomopathogenic fungi, are cosmopolitan in nature and are commonly present in soil (Kepler et al. 2015; Keyser et al. 2015; Rocha et al. 2013; Wyrebek et al. 2011). The ecological functions, population structure, and natural occurrence of these two most abundant genera have been studied more thoroughly through the use of molecular approaches over the last three decades (Bidochka et al. 1998; Kepler et al. 2015; Kessler et al. 2003; Keyser et al. 2015; Meyling et al. 2011; Muñiz-Reyes et al. 2014; Pérez-González et al. 2014; Rocha et al. 2013; Vanninen 1996). Using molecular techniques in order to delimitate entomopathogenic fungus species not only is a key tool in fungal systematics but also is of importance in biological controls of against pests, establishing policy in conservation biology and defining fungal diversity. Recently, it has been demonstrated that the two genera of *Beauveria* and *Metarhizium*

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are composed of morphologically indistinguishable cryptic species (Kepler et al. 2014; Rehner et al. 2011). The circumscription of the *Metarhizium* cryptic species is currently based on the previous criteria provided by Driver et al. (2000) and Bischoff et al. (2009), which have been updated based on specific genomic intergenic sequence markers (Kepler and Rehner 2013). However, the genus *Metarhizium* has been included within other genera in addition to the elevation of some *Metarhizium flavoviride* varieties to a species level based on a multilocus phylogeny (Kepler et al. 2014). The potential of entomopathogenicity of IPF, particularly *Metarhizium* and *Beauveria*, in agroecosystems has been evaluated by many different research studies (Meyling and Eilenberg 2007; Keyser et al. 2015). An extensive number of commercial formulations of *Metarhizium* and *Beauveria* have been developed for crop protection (Castrillo et al. 2011; de Faria and Wraight 2007).

Isolations of *Metarhizium* and *Beauveria* have been conducted extensively from soil samples of terrestrial ecosystems or phylloplanes of different crop systems around the world (Bidochka et al. 1998; Garrido-Jurado et al. 2015; Kepler et al. 2015; Meyling et al. 2011; Muñiz-Reyes et al. 2014; Nishi et al. 2011, 2015; Pérez-González et al. 2014; Rezende et al. 2015; Rocha et al. 2013; Vanninen 1996) in order to provide insight into the ecology and composition of indigenous populations of *Metarhizium* and *Beauveria* and provide a pool of novel fungal isolates for development into potentially new microbial control agents. Almost all studies considered the identification, biodiversity, and population dynamics of IPF within agricultural and managed ecosystems to develop a suitable candidate for integrated pest management in temperate, subtropical, or tropical agroecosystems. Despite all these efforts, little attention has been given to the natural occurrence of indigenous communities of *Metarhizium* and *Beauveria* in forest areas (Luan et al. 2013; Nishi et al. 2011, 2015; Nunes Rocha et al. 2009; Popowska-Nowak et al. 2016; Vanninen 1996). In insect pathology, the host-insect relation has long been believed to play role in the entomopathogenic fungal community structure. This traditional paradigm could be shifted toward their association with environmental tolerances; two genetically distinctive groups of the *M. anisopliae* lineage were characterized by thermal-active (agriculture area) and cold-active (forest region) growth (Bidochka et al. 2001, 2005), which were later identified as *Metarhizium robertsii* and *Metarhizium brunneum*, respectively (Bischoff et al. 2009). Similarly, *B. bassiana* has evolved in selected habitats, and any evidence of an insect-host-related population structure should be viewed primarily as coincidental and not as a result of co-evolution (Bidochka et al. 2002; Meyling and Eilenberg 2006, 2007; Meyling et al. 2009). Furthermore, more recent research shows that different cryptic species of IPF may also occur in the same environment (Pérez-González et al. 2014).

Forests are a dominant global biome, providing vital ecosystem services, habitat and economic value to humans, and a source of extraordinary biodiversity. Diverse forest communities can be affected by different factors including insect pest outbreaks. However, knowledge of the natural occurrence and biodiversity of entomopathogenic fungi is important to our understanding of forest ecosystems under the stress of disturbances such as drought, fire, insect outbreaks, deforestation, and climate change. Alternative control tactics that include microbial pesticides such as viruses, bacteria, and fungi that are environmentally friendly may establish viable biological control approaches. The main objective of the current study was to identify the initial distribution of soil-borne entomopathogenic fungi and the importance of environmental factors, particularly cold tolerance, for their occurrence in forest ecosystems so as to provide valuable information on natural distribution. We examined the ecology of indigenous *Metarhizium* and *Beauveria* isolates from the Foping National Nature Reserve (FNNR) in the Qinling Mountains located in southwest Shaanxi province, China.

Materials and methods

Study site and soil sampling

Soil sampling was conducted in three distinct altitudes ranging from 1070 m as a low elevation (107° 40′ 21″ E, 33° 34′ 12″ N), 2010 m as a middle elevation (107° 47′ 14″ E, 33° 39′ 79″ N) to 3000 m as a high elevation (107° 55′ 13″ E, 33° 45′ 81″ N) at the FNNR located on the Southern slopes of the Qinling Mountains in Shaanxi province, China (Fig. 1).

This area possesses a subtropical wet climate and thick vegetation (Wang et al. 2013). FNNR is a place of refuge for many species particularly the Giant Panda (*Ailuropoda melanoleuca*) and it stretches across the north subtropical and temperate zones. From the lowest elevations to nearly 2000 m, the canopy consists primarily of deciduous broadleaved *Quercus*. From 2000 to 2500 m, the mixture of coniferous and deciduous broadleaf forest is dominated by *Betula* and *Abies*. *Abies fargesii* dominates the canopy from 2500 to 2800 m (Du et al. 2011). The mean low temperature, − 0.1 °C, occurs in January, and the average highest temperature, about 26.6 °C, occurs mostly in July (http://www.chinaweatherguide.com).

A total of 60 soil samples were collected from the soil surface to a depth of 30 cm during July 20 to July 22, 2015. Within each altitude, 20 lines were selected. The orientation and position of each line were selected based on landform and safety of sampling. One single soil sample was prepared from five soil slices with approximately 10-m distance between two microsites, and each microsite was selected with inside diameter of about 1 m to take one soil slice (~ 50-m^2 total transect

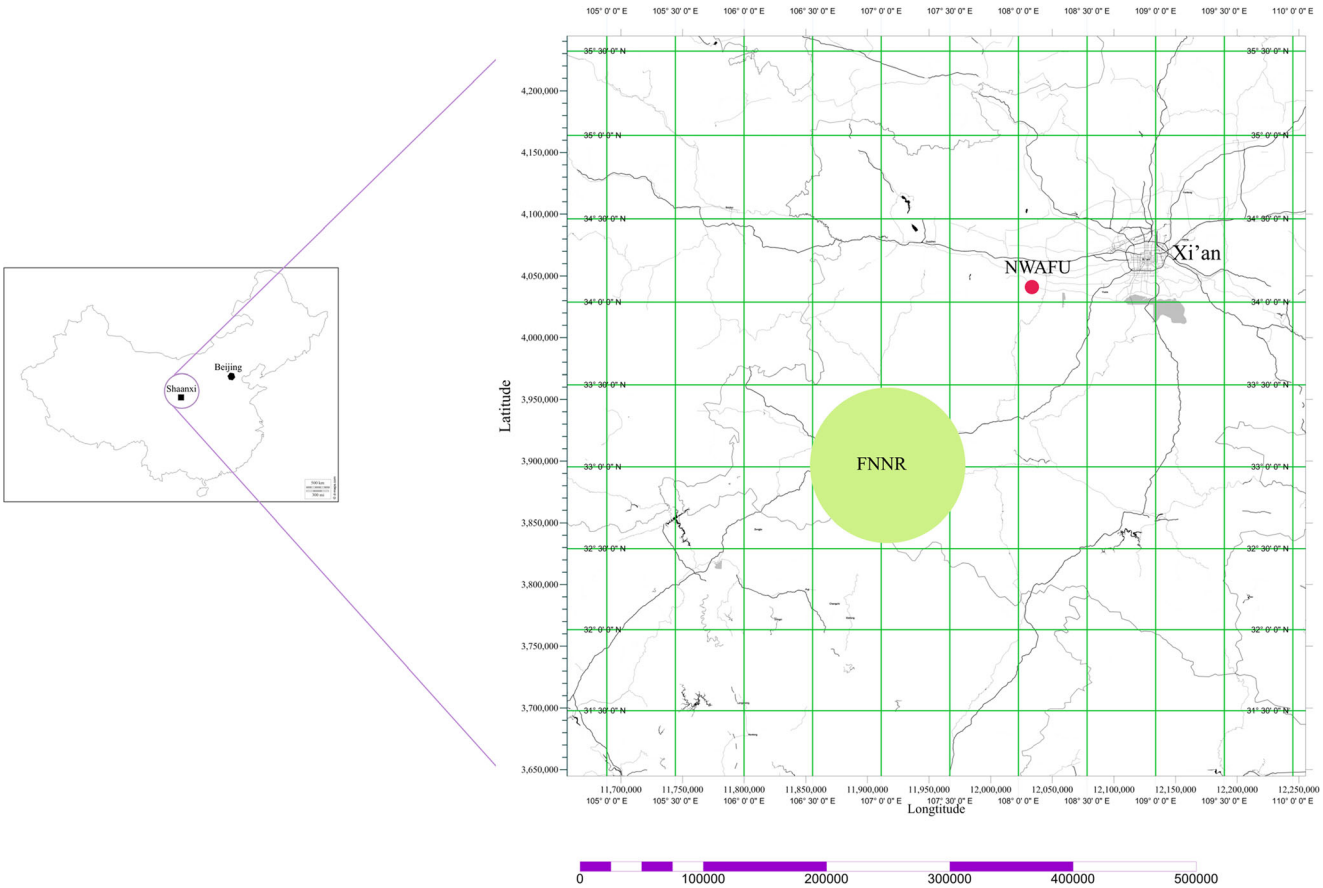

Fig. 1 *The map showing the geographical location of sampling site at the Foping National Nature Reserve (FNNR), Shaanxi, China.* At the sampled locality, three distinct altitudes were considered. At each altitude, 20 lines were selected with five soil cores within 50 m^2 with 10-m distance of each core approximately. The map of China was created from free maps available at d-maps.com (http://www.d-maps.com/pays.php?num_pay=77&lang=en) and the geographical map with coordinates was created by Surfer® 14 from Golden Software LLC (www.goldensoftware.com). *NWAFU* Northwest Agriculture and Forestry University, Xi'an: capital city of Shaanxi province

length). All soil cores for each transect were mixed and sieved (2-mm mesh) and 200 g soil was individually preserved from each line in a pre-labeled polyethylene Whirl-Pak® bag (ULINE, USA). Samples were transported on dry ice and maintained at a temperature of 4 °C at the Lab of Insect Related Resource, Institute of Entomology, Northwest A&F University (NWAFU), for processing within 7 days.

Isolation of insect pathogenic fungi (*Metarhizium* and *Beauveria*) by an in vitro semi-differential medium method

A semi-selective agar medium was utilized to isolate entomopathogenic fungi, as adapted from Posadas et al. (2012), and was composed of 10 g peptone l^{-1} (AOBOX, Beijing, China), 40 g D-glucose anhydrous l^{-1} (Solarbio, Beijing, China), 20 g agar l^{-1} (Biosharp, Anhui, China), 1 g yeast extract l^{-1} (OXOID, Basingstoke, Hampshire, England), 0.6 g cetyl trimethyl ammonium bromide l^{-1} (CTAB, Wolsen, Xi'an, China), 0.6 g streptomycin l^{-1} (MP Biomedicals, Shanghai, China), 0.3 g kanamycin l^{-1} (MP Biomedicals, Shanghai, China), 0.05 g tetracycline l^{-1} (Wolsen, Xi'an, China), and 0.01 g crystal violet l^{-1} (JHD®, Guangzhou, China). In order to isolate entomopathogenic fungi, a soil suspension was prepared from each soil sample as described by Kessler et al. (2003). Briefly, from each soil sample, 20 g of soil were added to a 250-ml Erlenmeyer flask including 100 ml sterilized distilled water and 1.8 g/l tetra sodium pyrophosphate (TSPP) to increase the possibility of soil particle separation; this mixture was homogenized for 3 h at 25 °C on a horizontal incubator shaker. One hundred microliters of homogenate was spread onto the medium as described above. All plates were incubated for 2 weeks at 25 °C in constant darkness. *Metarhizium* and *Beauveria* isolates were then identified by colony morphology. On this medium, *Metarhizium* and *Beauveria* colonies showed good growth of white aerial mycelium that was distinct against the violet background. We then selected those differing colonies with white mycelia and green conidia for *Metarhizium* and white mycelia and white conidia for *Beauveria*, and carried out confirmation with microscopic identification of conidial morphology. The desired colonies were aseptically transferred to one-quarter-strength SDAY (SDAY/4) (SDAY, 2.5 g/l

peptone, 10 g/l D-glucose, 2.5 g/l yeast extract, 20 g/l agar) using a sterilized toothpick. In total, 20 *Metarhizium* spp. and 2 *Beauveria* spp. isolates were obtained. All isolates were grown under ambient light/dark conditions at approximately 25 °C in the laboratory. Pure cultures were prepared by a single-spore isolation procedure (Choi et al. 1999) using continuous dilution of the conidia suspension of each isolate. Conidia of monosporic isolates from routinely outgrowing colonies were transferred into a 2-ml Eppendorf tube that included 20% sterilized glycerol in order to store them at − 80 °C until needed for assays.

Morphology of isolates

We assessed some morphological characteristics such as conidiation, radial growth rate, and general aspects (such as colony size, colony features) (Supplemental Table S1) for all *Metarhizium* isolates by pointing a 5-μl conidial suspension (10^7 conidia/ml of 0.02% tween 20) into the center of a Sabouraud dextrose agar plate (SDA, 10 g/l peptone, 40 g/l dextrose, and 15 g/l agar; 3-mm depth, ~ 15 ml) (Kamp and Bidochka 2002) for 15 days, at 25 ± 2 °C under a 10-h:14-h light:dark cycle, with three replicates for each isolate.

Fungal inoculations were permitted to grow on SDA medium for 48 h before starting the measurement. The apical extension rate of fungal hyphae (culture diameter) was determined as described by Ivey et al. (1996). In this case, the colony diameter was measured daily along the arrow lines marked at the back of the Petri-plate, from the center to the edge until 15 days of observation. Conidia were counted after the two mycelial plugs (2-cm diameter) were accurately taken halfway between the center and edge of the colony in opposite direction from each replicate (total for each isolate, six mycelial plugs) (Kamp and Bidochka 2002). We did not subject *Beauveria* isolates to morphological characterizations or bioassay experiments.

Genomic DNA extraction, PCR amplification, and sequencing

Total genomic DNA for *Metarhizium* and *Beauveria* was extracted from fresh mycelium as described by Glare et al. (2008) with some modifications. DNA concentration was estimated using a Nano-200 (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). All extracted DNA was stored at − 80 °C until used. For *Metarhizium*, partial sequences of two loci were amplified by PCR including the 5′ intron-rich region of translation elongation factor 1 alpha (5′-*TEF*) using the primer EF1T (5′-ATG GGT AAG GAR GAC AAG AC-3′) and EFjmetaR (5′-TGC TCA CGR GTC TGG CCA TCC TT-3′) (Bischoff et al. 2009) and a nuclear intergenic locus using the primer Mz_IGS3_1F (5′-CGT GGC TCC TGA CCA TGG TTG C-3′) and Mz_IGS3_4F (5′-GCG GGG GAG CCG ACT TGG A-3′) (Kepler and Rehner 2013). PCR amplifications were performed in a total volume of 50 μl, which included 5 μl 10X PCR buffer (10 mM Tris/HCl, pH 8.0, 50 mM KCl, 1.5–2.0 mM $MgCl_2$) (TaKaRa, Tokyo, Japan), 2 μl of 2.5 mM dNTPs (Roche Molecular System, Inc., Basel, Switzerland), 0.5 μl (2.5 U) rDNA *Taq* polymerase (TaKaRa Taq™), 10 pmol of each primer, 0.3 μl bovine serum albumin 0.1% (BSA) (TaKaRa) for each reaction, 2 μl of genomic DNA, and 37.6 μl sterilized Milli-Q H_2O. PCR cycle conditions for 5′-*TEF* were as follows: initial denaturation at 98 °C for 30 s, 38 cycles of 98 °C for 5 s, 58 °C for 20 s, and 72 °C for 1 min and final extension at 72 °C for 7 min. PCR amplification for the nuclear intergenic region was initiated by 95 °C for 2 min, then 40 cycles of denaturation, 30 s at 95 °C; annealing, 30 s at 62 °C; extension, 1 min at 72 °C; and final extension, 15 min at 72 °C. For phylogenetic placement of the *Beauveria* spp. isolates, partial sequences of the nuclear intergenic region *Bloc* and segment of *EF1-α* were selected (Rehner et al. 2006, 2011). An approximately 1000-bp fragment and a segment spanning part of *EF1-α* were amplified with the primer pair, 983F/2218R (Rehner and Buckley 2005). The spanning part of intergenic *Bloc* region approximately 1500 bp in length was amplified with the primer pair B5.1F/B3.1R (Rehner et al. 2006). The PCR reaction mixes were prepared as described above. PCR cycle conditions for *Bloc* were used as described by Rehner et al. (2006) and PCR was achieved for *EF1-α* using the thermal conditions reported by Johnson et al. (2009). The PCR assays were performed in an ETC 811 thermal cycler (Eastwin Life Science, Inc., Beijing, China). The PCR products were checked on 0.8% agarose gel and positive amplicons were purified with a BioSpin Gel Extraction Kit (Bio Flux™ Corporation, Tokyo, Japan). The purified PCR products were submitted to Invitrogen (Thermo Fisher Scientific, Beijing, China) for unidirectional Sanger sequencing with forward PCR primers. The nucleotide sequences were manually corrected using Chromas Pro (V. 2.0, Technelysium Pty Ltd). *Metarhizium* isolate sequences generated during the present study were deposited in GenBank (National Center for Biotechnology Information (NCBI)) under accession numbers KY286535 to KY286554 and KY286555 to KY286574, for *TEF* and intergenic spacer loci, respectively. Subsequently, the sequences of two *Beauveria* isolates (Qin 21 and Qin 22) have been deposited into GenBank under accession numbers KY593252, KY593253 (*EF1-α*), KY593254, and KY593255 (*Bloc* region). Single-pass sequence reads of *Metarhizium* were combined with *EF1-α* reference sequences from Bischoff et al. (2009) and the *Mz_IGS3* reference sequences from Kepler and Rehner (2013) and Kepler et al. (2015). Sequencing reads of *Beauveria* isolates were combined with sequences determined for taxonomically validated isolates from Rehner et al. (2011). Sequences of each gene were individually aligned using MAFFT ver. 7.305 (Katoh and Standley 2013) on

Extreme Science and Engineering Discovery Environment (XSEDE) with default parameters in CIPRES Science Gateway V.3.3 (Miller et al. 2010) (https://www.phylo.org/portal2/login!input.action). For *Metarhizium*, the resulting alignments of *EF1-α* and *Mz_IGS3* were trimmed to include ~800 base pairs and ~950 base pairs, respectively. The best-fit model of nucleotide substitution for each locus was determined with FindModel (Posada and Crandall 1998) (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). For the *EF1-α*, a F81model (Felsenstein 1981), with a gamma-distributed rate variation, was selected, and for the *Mz_IGS3*, K80 (Kimura 1980) with a gamma-distributed rate variation was suggested. For *Beauveria*, each of the two loci was trimmed to form final data matrices. The optimal substitution model for both *EF1-α* and *Bloc* was suggested by general time reversible (GTR), as implemented in FindModel. Finally, the resulting single-gene alignments were concatenated in the software Mesquite ver. 3.04 for Windows (Maddison and Maddison 2015) (http://mesquiteproject.org). A two-gene phylogeny of *Metarhizium* and concatenated *Beauveria* gene loci was derived with maximum likelihood (ML) estimation using PhyML under a determined substitution model, and the nexus files for ML analysis were generated through Mesquite ver. 3.04 (Maddison and Maddison 2015). The robustness of phylogeny was assessed by 500 locally running bootstrap replicates and executed from Topali ver. 2.5 (Milne et al. 2009). Finally, the resulting trees were printed using MEGA ver. 6.06 for Windows (Tamura et al. 2013). To analyze the intra- and inter-specific genetic variability of *Metarhizium* isolates, Kimura two-parameter (K2P) distances for 5′-*TEF* and *Mz_IGS3* were obtained using MEGA ver. 6.06 (Tamura et al. 2013). The intra- and inter-specific K2P distances of DNA sequences were visualized using box and whisker plots. All sequences of each gene were aligned using MAFFT ver. 7.305 (Katoh and Standley 2013) on XSEDE with the default setting as described above. FASTA file format was separately obtained using Mesquite ver. 3.04 (Maddison and Maddison 2015).

Experimental insects

To investigate the potential of our isolates for virulence, assays were undertaken in two series. First, we evaluated the pathogenicity of the isolates from the *M. robertsii* cluster (based on *Mz_IGS3* sequence region phylogenetic assessment) against the coleopteran model insect, *Tenebrio molitor* L. adult (yellow mealworm). Second, we conducted a bioassay to compare the pathogenicity of the two most virulent isolates from first screening with isolates of other species (in total, eight isolates).

Commercially supplied mealworm pupae were placed in a clear plastic box (19 cm × 12 cm × 5 cm, *L* × *W* × *H*) with wheat bran at 25 ± 2 °C, 55% RH, and a 16-h:8-h L:D photoperiod. When the pupae metamorphosed into adults, the beetle initially is light beige and then darkens and hardens to red, brown, and finally dark brown. We selected 1-week post-metamorphic beetles (red color) to evaluate the bioefficacy of *M. robertsii* isolates. Actively feeding fifth to sixth instars of *T. molitor* larvae (1.2 to 1.5 cm) were used in the second part of the bioassay. The last instar larvae were obtained commercially and reared on a mixture of wheat bran and flour until they reached the pupal stage. *T. molitor* pupae were collected from a stock colony and maintained in an incubator set at the same condition as mentioned above. Beetles were reared in the same laboratory conditions and the eggs were transferred to a clear plastic box (19 cm × 12 cm × 5 cm, *L* × *W* × *H*) on wheat bran to permit the eggs to hatch. Fifth and sixth instar larvae were selected for this bioassay.

In the final step, we evaluated the pathogenicity of the screened isolates with the least mean survival time (MST) against gypsy moth, *Lymantria dispar* L. (*Lepidoptera: Erebidae*), a serious defoliator of forests. The gypsy moth eggs were provided by the Research Institute of Forest Ecology and Environmental Protection, Chinese Academy of Forestry (CAF). Larvae that hatched from the gypsy moth eggs were reared to second instar at 25 ± 2 °C, 50% RH, and a 16-h:8-h L:D photoperiod in groups of 20 in 220-ml glass cups with plastic lids ventilated on one side through a 4-cm-diameter hole sealed with metal screen and larvae were provided an artificial diet.

Bioassay

Preparation of conidia

In order to prepare the suspension of *M. robertsii* conidia, single-spore isolates stored in 20% sterilized glycerol at −80 °C were grown on half-strength SDAY (~23-ml medium, 4-mm depth). These Petri plates were incubated for 2 weeks, without sealing them with parafilm and in total darkness at 25 ± 2 °C. They were then transferred to an incubator with 70% relative humidity for one more week with 12:12 L:D. Fresh conidia were harvested with a sterilized spatula at once, prior to use in sterile 0.02% Tween 20 (Tianjin Guangfu Fine Chemical Research Institute, Xi'an, China), and vortexed for 2 min. To reduce the negative influence of Tween, the fungal suspension stocks were diluted in sterilized ddH_2O. The concentration of suspensions was adjusted to 1×10^8 conidia/ml with a hemocytometer (Neubauer-improved hemocytometer, Lauda-Königshofen, Germany) under 20× magnification using an inverted research microscope (ECLIPSE TE2000-S, Nikon, Tokyo, Japan). The conidial germination rates of *M. robertsii* isolates were assessed by plating 100 μl of conidia in a 10^7 conidia/ml suspension onto quarter-strength SDAY at 25 ± 2 °C in total darkness (Inglis et al. 1997). The rate of germination in terms of percentage was calculated after

20 h with a count of a total 200 conidia per field of view based on a simple fraction *a*/*b*, where *a* and *b* are the number of germinated conidia and the sum of germinated and non-germinated conidia, respectively. This experiment was conducted in triplicate for each isolate. The mean value of three independent counts was used to obtain the percentage of germination. Viability was >95% for all *M. robertsii* isolates. The adults for *T. molitor* were completely immersed in a 15-ml fungal suspension (1×10^8 conidia/ml) with constant agitation. The control insects were treated with ddH_2O as described above. Having completed coverage of insects with fungal suspension, excess suspension was removed by placing the insects on a sterilized filter paper (Donyang Co., Ltd., Fushun, China). The 45 treated insects in three biological replicates were transferred to 24-well plastic boxes in which each well was ventilated with three holes (1.2-mm diameter). All the plastic boxes were arranged in a complete randomized design and maintained at standard environmental chamber conditions (25 ± 2 °C, 75 ± 7% RH, and 12-h:12-h L:D photoperiod). Insects were fed with wheat bran during a bioassay. Data were collected every day for 7 consecutive days. Insect cadavers were removed to be sterilized in 24-well plastic boxes containing filter papers that were moistened with sterilized ddH_2O to promote mycosis. The pathogenicity of *Metarhizium* species from other clusters, two *Metarhizium brunneum* (Qin 16, Qin 17), three *Metarhizium anisopliae* isolates (Qin 09, Qin 13, Qin 14), one *Metarhizium pingshaense* (Qin 18), and two *M. robertsii* isolates (Qin 06, Qin 08) from the first bioassay section, was assessed as described by Chen et al. (2014). Fungal conidia were harvested using a sterile spatula by gently scraping the surface of the 21-day-old sporulating culture of *Metarhizium* isolates and spores were transferred to a sterile 50-ml Falcon conical tube (GLT™-Comb, Shanghai Kirgen Co., Ltd., Shanghai, China). The conidial powder was weighed and mixed with sterile wheat bran substrate. The conidial concentration (2×10^8 conidia/g) and moisture contents of substrates (~45%) were adjusted with a hemocytometer determining the difference between the wet and dry weight of the wheat bran substrate mixture respectively. The conidial germination rates of *Metarhizium* isolates were separately determined as previously described (Chen et al. 2014). Six grams of each given mixture and 30 *T. molitor* larvae (fifth to sixth instar) were transferred to a sterile 240-ml round glass jar tissue culture with plastic screw lids (Xuzhou Shengshi Glassware Co., Ltd. Jiangsu, China). All the glass jars were placed in an environmental chamber set at 25 ± 2 °C, 55 ± 7% RH, and 12-h:12-h L:D photoperiod. Insect mortalities were observed daily for 15 days post inoculation with a fungus. Insect cadavers were individually moved into 24-well plastic boxes containing moist filter papers and incubated in a standard chamber under the same conditions as described above to promote sporulation and fungal outgrowth. The experiment was conducted with three biological replicates and organized as a completely randomized design. The concentration of 2×10^7 conidia/ml was used to evaluate the pathogenicity of the ultimate isolate (Qin 08) that was screened in the two-step bioassay. The second instar gypsy moth larvae were completely immersed in a 10-ml fungal suspension (2×10^7 conidia/ml) for 30 s with constant agitation. All the remaining bioassay procedures including control insect treatment are the same as described above for *T. molitor*.

Cold activity on conidial germination

The fungal isolates were cultured on SDAY medium in 90-mm Petri plates. The cultures were incubated as mentioned above. The conidia of the 20 *Metarhizium* isolates were collected with the aid of a sterile spatula and suspended in 8 ml sterile distilled water in a sterile 50-ml Falcon tube. After vigorously agitating the fungal suspension with a vortex for 1 min horizontally, samples were adjusted to a 10^5 conidia/ml concentration using an advanced hemocytometer. An aliquot of a 20-μl conidial suspension of each isolate was placed (without spreading) on 4 ml PDAY plus 0.001% (*w*/*v*) benomyl (50% active ingredient; Jiangsu Lanfeng Biochemical Co., Ltd., Jiangsu, China) (Braga et al. 2001; Santos et al. 2011) in the center of a Petri plate (35 × 10 mm) according to the amended Braga et al. (2001) method. The plates were immediately placed on a tray covered with aluminum foil, kept at 5 ± 1, 10 ± 1, 15 ± 1, and 20 ± 1 °C according to the Fernandes et al. (2010) method. Germination was counted at days 15, 15, 7 and 2 after inoculation, respectively. Control conidial suspensions were not exposed to cold but inoculated on PDAY-B and held at 25 ± 1 °C in total darkness. The conidial germination was observed 20 h after inoculation. This experiment was assessed using a completely randomized design and triple replicates for each isolate, including controls.

Deposit of fungal strain in public service collection

The isolate Qin 08 (*M. robertsii*) was deposited in the China General Microbiological Culture Collection Center (CGMCC) (http://www.cgmcc.net) with the designation CGMCC 7.286.

Statistical analysis

All the experiments were performed using three biological replicates for each isolate. The test of cold effect on germination was repeated on two different dates over a period of 2.5 months. Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test (OriginLab, Northampton, MA). Statistically significant differences among the studied isolates were

established using the analysis of variance (ANOVA), followed by a post hoc Tukey-Kramer test. All statistical tests were carried out using the SAS JMP statistical program ver. 13.2.0 (SAS Institute Inc., Cary, NC, USA). Mean survival times (MSTs) were determined using the OASIS 2 online tool (Han et al. 2016). All graphs were produced using Prism 6.0 for windows (GraphPad, La Jolla, CA, USA, http://www.graphpad.com and edited using Xara Photo & Graphic Designer for Windows (Xara Group Ltd., London, UK, www.xara.com). *P* values of less than 0.05 were considered statistically significant.

Results

Metarhizium occurrence and DNA analysis

A total of 20 *Metarhizium* and 2 *Beauveria* isolates were isolated from the soil samples collected at three different elevations. The intermediate elevation yielded the highest number of *Metarhizium* isolates ($n = 9$), whereas eight and three isolates were obtained from low- and high-elevation soils, respectively. The frequency distribution of *Metarhizium* isolates by elevation was statistically homogenous ($\chi^2 = 3.103$; df = 2; $P = 0.1717$). Two *Beauveria* isolates were isolated from the intermediate elevation. All the *Metarhizium* isolates were identified as belonging to the *M. anisopliae* s.l. complex based on their characteristic colony color and conidial shape. In order to confirm species identity, all *Metarhizium* isolates were sequenced for the 5′ intron-rich elongation factor 1 alpha (*EF1-α*) gene and the intergenic region *Mz_IGS3*. PCR amplicons for the 5′-*EF1α* gene ranged from 804 (Qin 10) to 824 bp (Qin 11) with an average length of 812 bp. The maximum likelihood (ML) phylogeny was inferred from a 5′-*EF1α* sequence alignment (MAFFT ver.7) (Katoh and Standley 2013) based on an 800 character alignment comprising a total of 31 sequences, including the 20 Qinling Mountains isolates and 11 reference strains (Bischoff et al. 2009) (Fig. 2(a)).

Eighteen of our isolates clustered together within the *M. robertsii* clade with bootstrap support of 94%, after which 2 different sub-clades and 14 genotypes were identified (Fig. 2(a)). The two remaining isolates (Qin 16 and Qin 17) formed a single group with *M. brunneum* based upon a strong bootstrap value of 96%, after which two different sub-clades with two genotypes were delineated. Morphologically, these two isolates differed, particularly in colony color and colony features (Supplemental Table S1). PCR-amplified products of the *Mz_IGS3* produced a range of fragment sizes from 1051 (Qin 05) to 1194 bp (Qin 19) with an average length of 1114 bp. The ML phylogenetic tree of the *Mz_IGS3* was deduced as described for 5′-*EF1α* with known related sequences (ten reference sequences) (Kepler and Rehner 2013; Kepler et al. 2015) grouping our isolates into four different clades, including *M. brunneum*, *M. anisopliae*, *M. pingshaense*, and *M. robertsii* with high bootstrap support (100%) (Fig. 2(b)).

In fact, the *Mz_IGS3* phylogeny revealed that all *Metarhizium* isolates clearly clustered to a PARB clade (Bischoff et al. 2009). More specifically, the 14 *Metarhizium* isolates clustered with *M. robertsii*, which further clustered as 3 different sub-clades with strong bootstrap support (92%). Furthermore, nine different genotypes were identified within *M. robertsii* sub-clades (Fig. 2(b)). Our study highlights how *M. robertsii* isolates were the predominant species across three distinct elevations. *M. brunneum* and *M. anisopliae* isolates were isolated only from low and middle elevations. On the other hand, *M. pingshaense* was isolated only from high elevations. Moreover, information from our phylogenetic analysis revealed 2, 3, 1, and 12 genotypes within *M. brunneum*, *M. anisopliae*, *M. pingshaense*, and *M. robertsii*, respectively (Fig. 2(b)). Subsequently, two genotypes were observed within *B. bassiana* isolates based on the *Bloc* region and *EF1-α* sequences (Fig. 3).

Average K2P distances between and within species were 2.4 and 0.5% for the *EF1-α* gene, respectively, and had 4 and 1.8% inter- and intra-specific variation for the *Mz_IGS3* (Fig. 4).

Morphology of isolates and culture studies

Morphological characterizations of *Metarhizium* colonies suggest that the 20 *Metarhizium* isolates belonged to the *M. anisopliae* s.l. complex and the two isolates of *Beauveria* belonged to *B. bassiana* s.l. Most colony features of *Metarhizium* isolates were smooth with a regular yellow colony pattern (Fig. 5, Supplemental Table S1) while others were irregular.

After inoculation at 27 ± 2 °C for 8 days, four isolates (Qin 04, Qin 06, Qin 08, and Qin 09) occupied the entire Petri plate surfaces. We consider this growth rate to form a large colony (> 80 mm in diameter), while a growth rate < 70 mm is considered as a small colony. Moreover, the extensive phenotypic variability of the *Metarhizium* isolates on SDA medium was also found clustered in analyses with the Mesquite software ver. 3.02 for Windows (Maddison and Maddison 2015). We evaluated the different features (26 morphological characters) of *Metarhizium* spp. colony isolates on the medium as some morphological characterizations (Supplemental Table S1). Morphologically based clustering is composed of two main clades (A and B) in which they divided into three sub-clades (Fig. 6).

An incongruence was observed between the morphology and the molecular phylogeny. For instance, Qin 09 (*M. anisopliae*), Qin 06, and Qin 08 (*M. robertsii*) grouped in sub-cluster A1, although they are different species according to the molecular phylogeny (Fig. 2(b)). Qin 13 and Qin 14

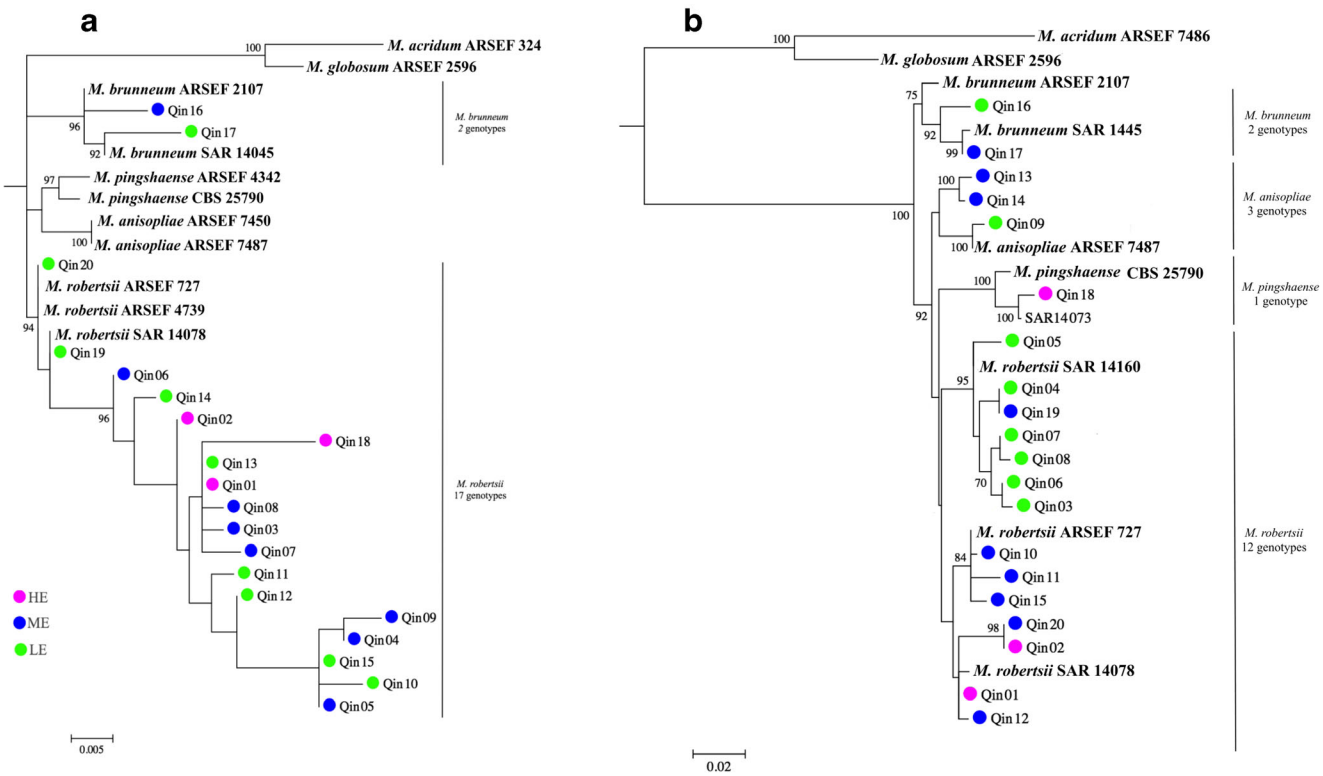

Fig. 2 *Maximum likelihood (ML) phylogenetic tree of 5′- EF1-α* ***(a)*** *and nuclear intergenic region Mz_IGS3* ***(b)*** *sequences of Metarhizium strains.* **a** This tree is rooted using *Metarhizium acridum* and *Metarhizium globosum* as outgroups. Reference sequences were taken from the study of Bischoff et al. (2009) and Kepler et al. (2015). Bootstrap values are based on 500 pseudo-replicates; values > 70% are shown. **b** The phylogram was rooted using *M. acridum* and *M. globosum* as outgroups. Representative strains were taken from the study of Kepler and Rehner (2013) and Kepler et al. (2015). Maximum likelihood bootstrap support values are given at the nodes and values ≥ 70% are shown. In both phylogenetic trees, the scale bar represents the number of expected changes per site. ARSEF (USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA), CBS (Centraalbureau voor schimmelcultures Fungal Biodiversity Center, Utrecht, the Netherlands), and SAR (laboratory collection of Dr. Stephen A. Rehner, Beltsville, MD, USA) numbers are in bold. *HE*; high elevation, *ME*; middle elevation, *LE*; low elevation

(*M. anisopliae*) grouped together but clustered with other *M. robertsii* strains as is shown by the sub-clade B2. Similarly, Qin 16 and Qin 17 (*M. brunneum*) grouped together but clustered with Qin 04 (*M. robertsii*) (sub-clade A3) and Qin 18 (*M. pingshaense*) clustered with *M. robertsii* strains (sub-clade B3).

Growth assessment and in vitro conidiation study

A significant difference among all *Metarhizium* isolates in growth rate (mm/day) was found at 25 ± 2 °C (Fig. 7: $F_{19, 59} = 1094.61$, $P < 0.0001$). The maximum growth rate was 80 mm/day achieved by the Qin 06, Qin 08, and Qin 09 isolates within 8 days on SDA medium. The minimum growth rate observed was 24 mm/day, calculated for Qin 10. Thus, we analyzed 8 days of growth rate data. However, the colony diameters were recorded daily during 15 days of growth. In vitro conidiation was determined after 15 days at 25 ± 2 °C (Fig. 7).

Conidial production was highly significant among all the isolates ($F_{19, 119} = 32.72$, $P < 0.0001$). Qin 08 gave the highest conidial yield of 4.3×10^7 conidia/cm^2 of the medium. However, there were no significant differences in conidial production between Qin 08 and Qin 06, Qin 09, Qin 14, and Qin 16. Conversely, the conidial yield of Qin 19 tended to be lower than that of the other isolates but Qin 19 did not differ significantly from Qin 01, Qin 02, Qin 03, Qin 04, Qin 05, Qin 07, Qin 10, Qin 11, Qin 12, Qin 13, Qin 15, Qin 17, Qin 18, and Qin 20 in conidial production.

Virulence

All isolates grouped in the *M. robertsii* cluster used in the initial screening bioassay were pathogenic to *T. molitor* adults ($F_{13, 41} = 207.26$, $P < 0.0001$) with differential virulence. As a result, two *M. robertsii* strains (Qin 06 and Qin 08) were selected in terms of Tukey's grouping (data not shown). In the subsequent bioassay, differences were found in mean survival times (MSTs) for each isolate based on daily mortality readings of yellow mealworm larvae with a single conidial concentration (2×10^8 conidia/g) among the eight isolates. The MSTs of yellow mealworm larvae when indirectly treated (mixture of wheat bran substrate and fungal suspension) with

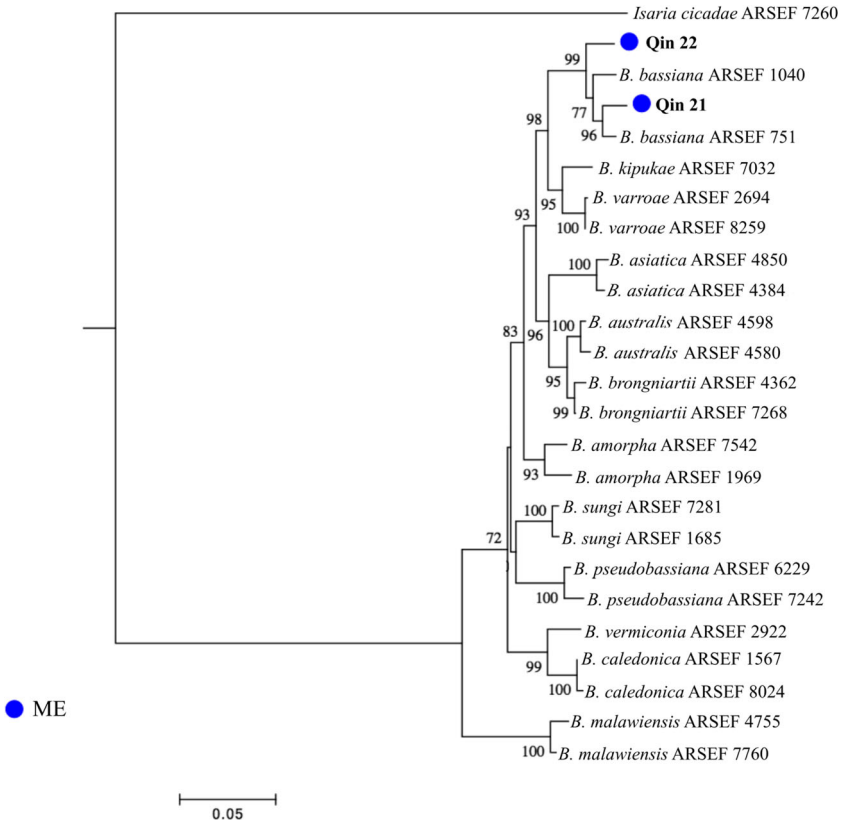

Fig. 3 *Maximum likelihood tree inferred from concatenation of the Bloc region and EF1-α sequences of Beauveria spp. using the GTR (general time reversible) + gamma model substitution.* The tree is rooted using *Isaria cicadae* as the outgroup, and branch lengths represent evolutionary distance. Numbers at the nodes indicate a PhyML bootstrap higher than 70%. The two *B. bassiana* isolates isolated with in vitro selective medium in this study are in bold lettering. ARSEF (USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA). *ME*; middle elevation

2×10^8 conidia/g displayed a different ranking in pathogenicity (Supplemental Table S2).

Isolate Qin 08 could kill the larvae faster than the other isolates, with an MST of 5.54 days. In comparison with Qin 09, Qin 16, and Qin 17, Qin 13 appeared to kill the larvae even faster; however, this difference was not significant. The rest of the MSTs of the *Metarhizium* spp. isolates ranged from 7.93 to 8.33 days and did not differ in pathogenicity (Tukey test, $P < 0.0001$).

Pathogenicity against *L. dispar*

The first infected larva died within 4 days after exposure to *M. robertsii* conidia (Qin 08). Cumulative mortality increased in the following days and reached 39% (± 1.6%, ±SD) after 14 days; all control larvae survived. Mycelium and conidia, respectively, of the *Metarhizium* isolate developed on dead larvae after 4 and 7 days of incubation on cadavers in an environmental chamber with saturation humidity (Fig. 8(B)).

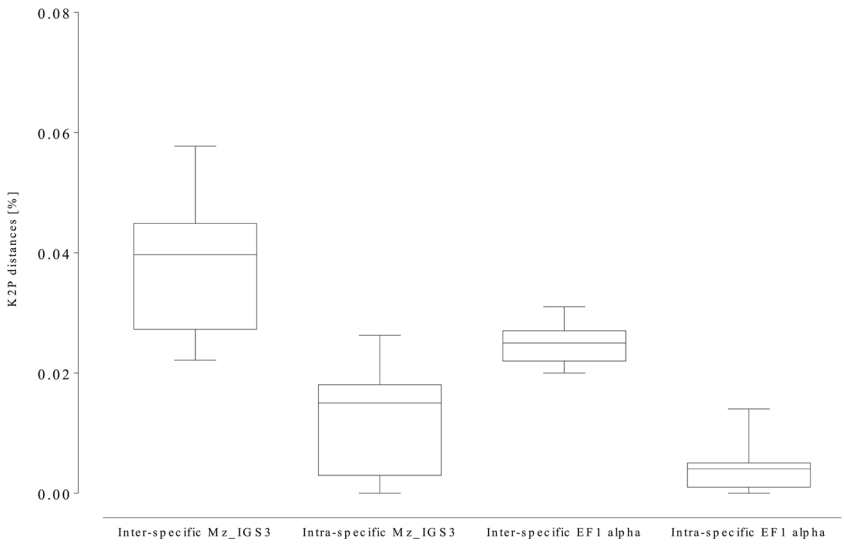

Fig. 4 *The distribution of intra- and inter-specific genetic divergences for the EF1-α and Mz_IGS3 gene fragments.* The lower and upper boxes represent the first and third quartile, respectively; their intersection is the median, and the whiskers represent the minimum and maximum K2P distances

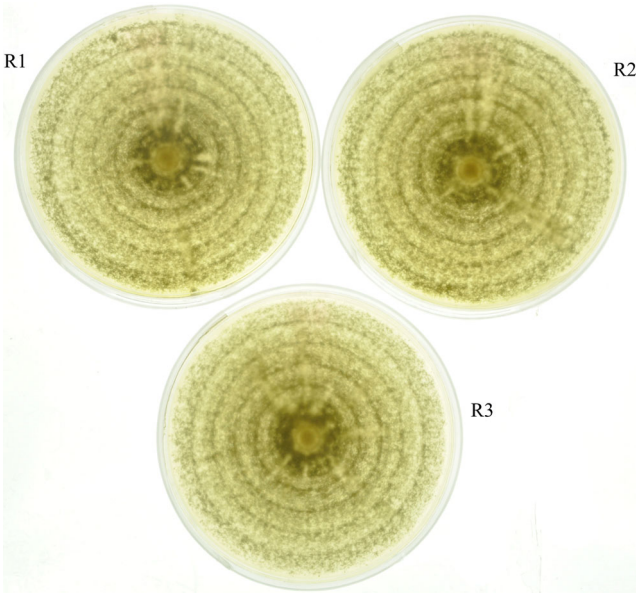

Fig. 5 *M. robertsii (isolate Qin 06) colony pattern formation.* The picture shows the growth pattern of isolate Qin 06, concentric rings, after 8 days inoculating the fungal conidial suspension (5 μl of 1×10^5 conidia/ml) in the center of a Petri plate with three replications on SDA medium. In order to display the fungal colony pattern formation, the photo has been carefully taken from the inverse side of the Petri plates with backlight using mounted LED flat panel light (Shenzhen Superolux Lighting Co. Ltd., Shenzhen, Guangdong, China)

Cold activity

Conidial germination response in the range of temperatures (5 ± 1, 10 ± 1, 15 ± 1, 20 ± 1, and 27 ± 1) differed widely among the 20 *Metarhizium* spp. isolates at 15-day, 7-day, 2-day, and 20-h post-inoculation, respectively (Supplemental Table S3 and Fig. 6).

The impact of temperature on germination rate (%) was highly significant for all temperatures ($F_{19,\ 59} = 85.79$, $F_{19,\ 59} = 17.36$, $F_{19,\ 59} = 10.07$, $F_{19,\ 59} = 10.26$, and $F_{19,\ 59} = 4.45$ at 5 ± 1, 10 ± 1, 15 ± 1, 20 ± 1, and 27 ± 1, respectively). The conidial germination of isolates was significantly reduced when conidia were exposed to 5 ± 1 for 15 days. Based on the results, Qin 04 from low elevation (85.28%) and Qin 18 from high elevation (45.69%) were markedly more resistant to low temperatures than the other isolates at the same temperature. Furthermore, high variability was observed when conidia were exposed to 10 ± 1 for 15 days, with results ranging from 10 (Qin 06) to 86.29% (Qin 04) of relative germination. No correlation was found between elevation of origin of *Metarhizium* isolates and temperature ranging from 5 to 20 °C (data not shown). A Tukey-Kramer paired-wise comparison test showed that there was a significant difference between biological attributes (colony growth rate, conidiation, and cold activity) of the *Metarhizium* isolates from low

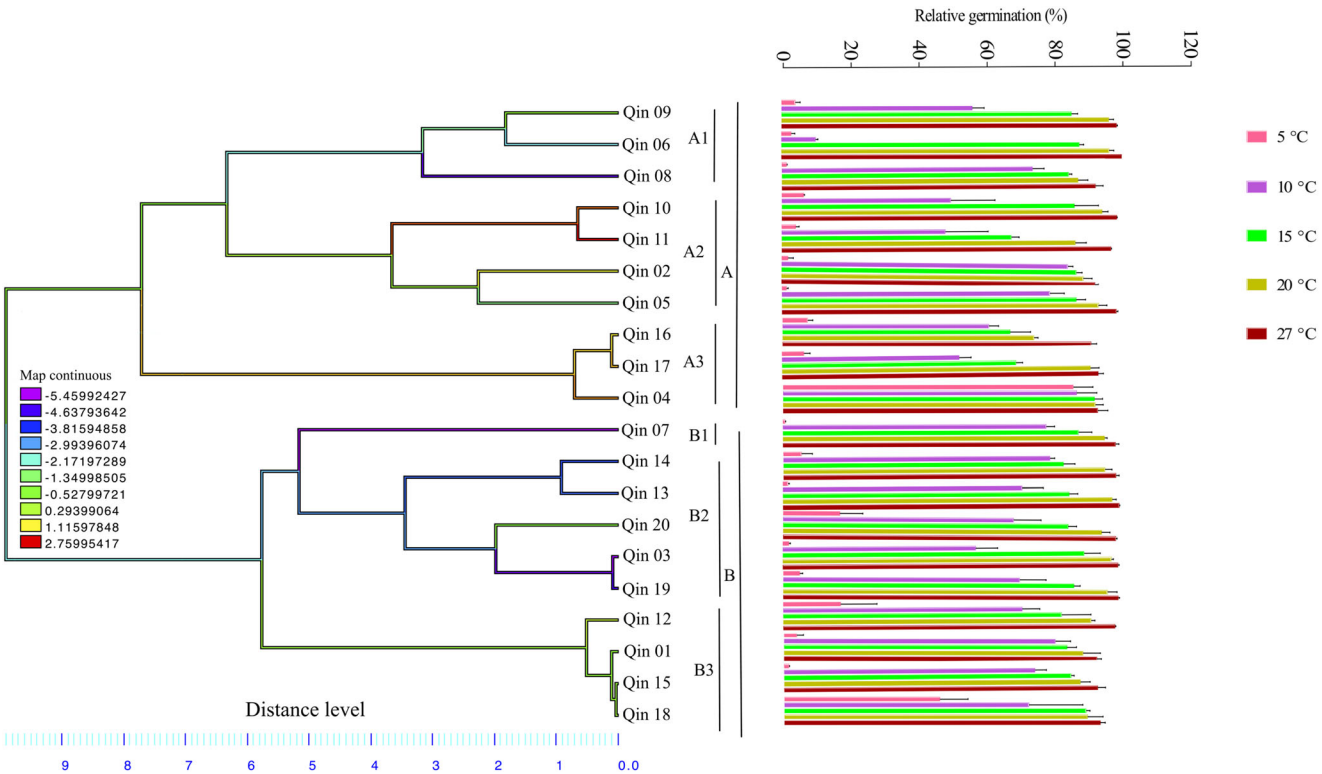

Fig. 6 *Hierarchical cluster of 20 Metarhizium spp. isolates (terminal taxa).* The clustering is based on 26 morphological characters (Supplemental Table S1) and the germination rate of conidia from each isolate in cold conditions ranging from 5 to 20 °C (Supplemental Table S3, control; 27 °C). Dendrogram obtained by simulated tree module with uniform speciation with sampling method in Mesquite software ver. 3.11

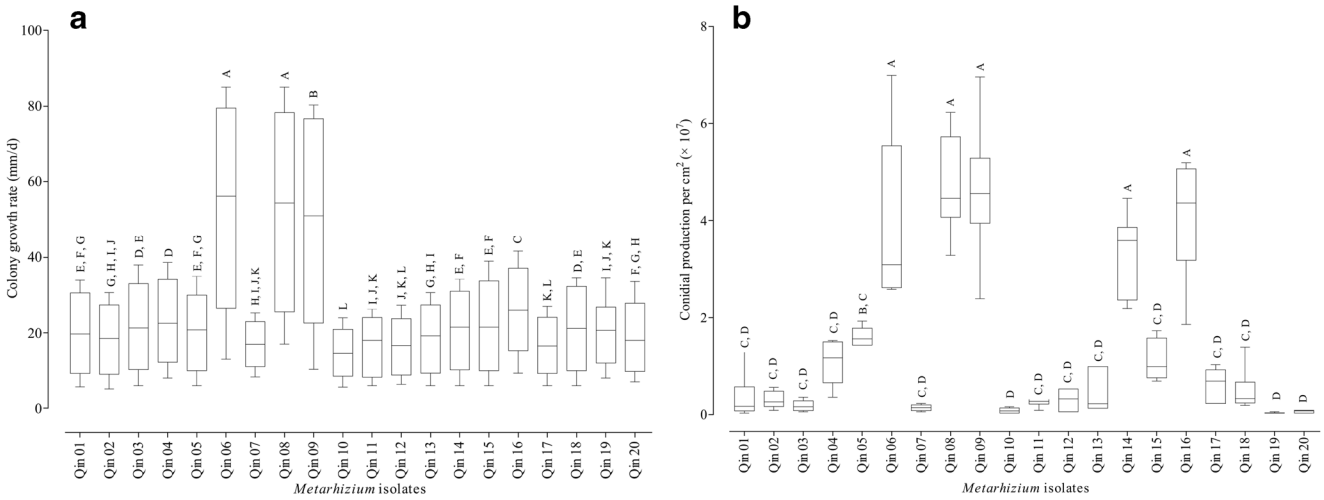

Fig. 7 *Colony growth rate and conidial production of Metarhizium spp. isolates on SDA medium.* A Average of growth rate throughout of 8 days ($F_{19,\ 59} = 1094.61$, $P < 0.0001$); B conidiation of isolates after 15 days ($F_{19,\ 119} = 32.72$, $P < 0.0001$); the box and whisker plot displays IQR, interquartile range (lower and upper quartile and median). The means of radial growth rate and conidiation of isolates were statistically evaluated with Tukey-Kramer HSD test. Multiple comparisons were significant ($\alpha = 0.05$); means with the same letters are not significantly different

elevations compared with intermediate elevations ($P = 0.0248$). However, there were no significant differences between biological attributes of the *Metarhizium* isolates from high elevations with intermediate elevations ($P = 0.1710$) and from high elevations ($P = 0.9778$).

Discussion

Categorizing variant strains of soil-borne entomopathogenic fungi with sequencing can provide a fine-grained view of their diversity in the soil (Kepler et al. 2015; Keyser et al. 2015; Meyling et al. 2011; Muñiz-Reyes et al. 2014; Nishi et al. 2011, 2015; Rezende et al. 2015). This study is the first to document the natural distribution of IPF from different distinct elevations that identified indigenous insect pathogenic fungal strains (*Metarhizium* and *Beauveria*) in the Qinling Mountains at FNNR. The use of a semi-selective isolation medium explicitly documented the different potentials in entomopathogenicity of *Metarhizium* isolates against mealworm adults and larvae. These variabilities would not likely be seen, if we had used an insect bait method. In other words, the insect bait method is not often to be highly susceptible to all insect pathogenic fungus species or isolates (Muñiz-Reyes et al. 2014). Phylogenetic placement of the *Metarhizium* isolates based on the 5′-*TEF* region identified two species, *M. brunneum* and *M. robertsii* (Fig. 2(a)), but a phylogenetic tree based on *Mz_IGS3* revealed four species: *M. anisopliae* (Qin 09, Qin 13, and Qin 14), *M. brunneum* (Qin 16 and Qin 17), *M. pingshaense* (Qin 18), and *M. robertsii* (14 isolates) (Fig. 2(b)). Although *TEF* distinguished *M. brunneum* and *M. robertsii* with a well-supported bootstrap, *TEF* failed to differentiate indigenous isolates of *M. anisopliae* and *M. pingshaense* from *M. robertsii* isolates. Compared with *TEF* results, the relationship between species in the PARB clade was highly supported using the intergenic locus, with all internal branches having a bootstrap value of 100%, and having more than 70% for *M. anisopliae*, *M. pingshaense*, and other species respectively. Based on our data, higher intra- and inter-specific *Mz_IGS3* K2P distances were observed in nucleotide sequences than in analysis of 5′-*TEF* sequences among *Metarhizium* species (Fig. 4), demonstrating the high potential of *Mz_IGS3* for *Metarhizium* species delimitation of closely related species in the PARB clade. Some phylogenetic studies from Brazil reported 5′-*TEF* failed to clearly cluster some *Metarhizium* isolates with known species in the PARB clade (Lopes et al. 2013a, b). Rezende et al. (2015) reported the inability of the *Mz_IGS3* locus to replicate the consensus phylogeny for Brazilian indigenous *Metarhizium* isolates in the PARB clade compared with 5′-*TEF* and *Mz_FG543i* sequence regions. Nevertheless, the three-locus concatenated markers yielded a highly resolved and well-supported phylogeny of the PARB clade. In this study, the concatenation of two loci also unambiguously differentiated four closely related *Metarhizium* species in the PARB clade (Fig. 9).

In addition, all the four *Metarhizium* species and *B. bassiana* isolates are distinct from the ex-type sequences that were included as taxonomic reference vouchers. This high level of intra-clade diversity under a strict genealogical species concept, which defines species boundaries on the basis of genealogically exclusive clades (Taylor et al. 2000), can potentially be regarded as existence of different species. Such intra-specific lineages might be due to widespread recombination between species, which allows incompatible alleles to arise between species and it can be delineated with DNA typing (Kepler et al. 2015). *M. robertsii* s.l. was the abundant

Fig. 8 *Symptoms of T. molitor larvae and pupae, and beetle and gypsy moth larvae infected by M. robertsii (Qin 08).* Note the exudate droplet formation on mealworm (black arrow; a5, a6), on gypsy moth larvae (b6), and on a PDAY (D) by 15-day-old *M. robertsii* (Qin 08) cultures. Droplets on mealworm and gypsy moth are colorless and intensely dark, respectively. The droplets derived from PDAY are brownish. Visible infection process of *M. robertsii* on a gypsy moth including the white mycelium formation on the surface of the cadaver (b1), consequently, hyphal extrusion and conidiation with a different intensity of color ranging from slightly yellow (b2), yellow (b3), light greenish (b4 and b5) to dark green (b6). These same processes have been observed in mealworm larvae and pupae and adult beetles during the fungal infection including mycosis with a white color (a1, a3, and a7) and conidial production on cadavers with a green color (a2, a4, and a8). The specimen figures are taken by the advanced stereo microscope system (Discovery V20, Zeiss; CCD, Axio ICc5, Zeiss; and Leica 205C, Germany and Canon 7D mark2, Japan)

species group isolated from soil samples across the elevation gradient that were collected from low to high elevations. *M. anisopliae*, *M. brunneum*, and *M. pingshaense* were evidently rare within this community. Our results are in agreement with other studies showing that a single species of *M. robertsii* was the predominant species from the *M. anisopliae* complex, and two *Beauveria* isolates phylogenetically clustered with *B. bassiana* species (Nishi et al. 2011; Ormond et al. 2010; Rezende et al. 2015). Although we found that *M. robertsii* was distributed from low to high elevations, our study does not allow us to conclude whether this natural distribution is a result of the plant type alone (Wyrebek et al. 2011) or is also influenced by other biotic and abiotic influences such as temperature, availability of insect hosts, soil type, soil moisture content, and/or other physiochemical soil properties (Medo and Cagáň 2011). In insect pathology, *Metarhizium* spp. is indisputably regarded as a thermophile at the species level (Fernandes et al. 2008; Vanninen 1996). And the abundance of species such as insects and plants decreases with increasing elevation because of the reduction in temperature (Qian and Ricklefs 2016). In our study, this may explain the overall lower frequency of the *Metarhizium* species in high elevations. The canopy at FNNR is dominated with coniferous trees at high elevation (Du et al. 2011). In addition, conifer needles help create acidic soil conditions that not only influence the kinds of vegetation that can grow, but also have an

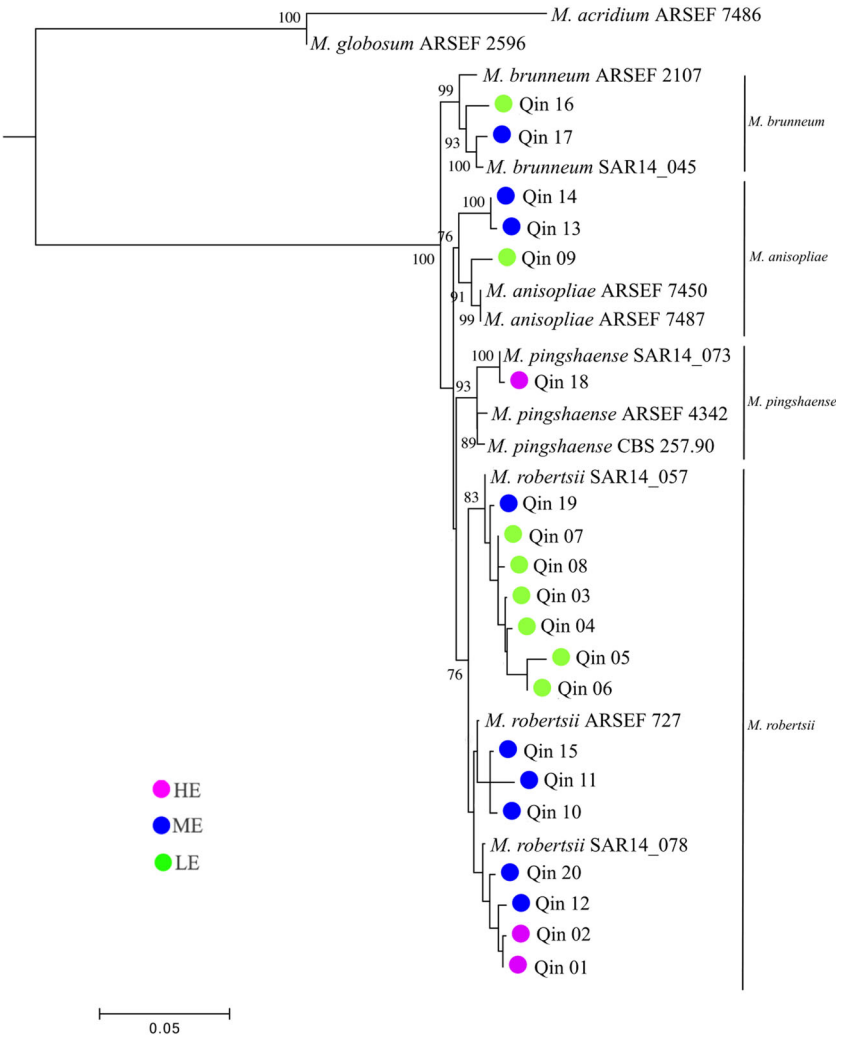

Fig. 9 *Maximum likelihood phylogeny of the combined data set of 5'- EF1-α and Mz_IGS3 sequences for 20 Chinese Metarhizium isolates.* The tree was rooted using *M. acridum* and *M. globosum* as outgroups. Support values greater than 70% are shown. The scale bar represents the number of expected changes per site. ARSEF (USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA), CBS (Centraalbureau voor schimmelcultures Fungal Biodiversity Center, Utrecht, the Netherlands), and SAR (laboratory collection of Dr. Stephen A. Rehner, Beltsville, MD, USA). *HE*, high elevation; *ME*, middle elevation; *LE*, low elevation

effect on microbial communities (Reich et al. 2005). Here, we speculate that the isolates (Qin 01 and Qin 02: *M. robertsii* and Qin 18: *M. pingshaense*) from high elevations might be able to withstand the acidic soil and/or have a positive interaction with the conifer rhizosphere metabolites. This is consistent with the potential for *Metarhizium* to grow across a broad pH spectrum (2.5–10.5) and ability to modulate the pH of its immediate environment through the production of ammonia (Hallsworth and Magan 1996). Our results are consistent with studies that also showed that *Beauveria* species are infrequent in forest soils (Popowska-Nowak et al. 2016; Vanninen 1996). In contrast, Chandler et al. (1997) and Medo and Cagáň (2011) found that *B. bassiana* and *Isaria farinosa* were more frequent than *M. anisopliae* in forest habitats. A satisfactory explanation for the discrepancy between the *Metarhizium* isolate frequency of distribution and the *Beauveria* isolate occurrence might be related to the resistance of *Metarhizium* conidia to the harmful abiotic factors and/or biodegradation in the biologically active soils of moist forest sites (Vanninen 1996). However, in our study, we did not measure the physical and chemical properties of soil samples or the pH of soil. To draw a definitive conclusion about the high frequency of *Metarhizium* and low frequency of *Beauveria* in the forest ecosystem, further investigations with regular and seasonal soil sampling, as well as the physical and chemical characterization of soil samples, are required to understand the interactions of these fungi with other organisms in these ecological niches.

Estimates of MST values show that Qin 08 (*M. robertsii*) is more virulent against yellow mealworm adult and larvae and gypsy moth larvae than the other isolates (Supplemental Table S2). On the other hand, Qin 16 (*M. brunneum*) with a high MST value (9.87 days) shows less virulence against yellow mealworm larvae. Qin 06 (*M. robertsii*) and Qin 18 (*M. pingshaense*) differed in virulence from three isolates namely Qin 09 (*M. robertsii*), Qin 13 (*M. anisopliae*), and Qin 17 (*M. brunneum*). Low MST values can reduce the host's time to mobilize defenses and resist infection. Thus, the Qin 08 strain shows promise as a candidate for biological control. Considering the findings of phylogenetic analysis, along with

the initial pathogenicity assay results, Qin 06 and Qin 08 as pathotypes seem to be included in the same sub-clade. This may indicate the relationship between virulence and phylogenetic clustering between the tested isolates.

Our study also showed significant differences in conidiation and in the radial growth rate of different *Metarhizium* spp. isolates. Our data demonstrated that in vitro conidial production and the growth rate on SDA medium of Qin 06, Qin 08, and Qin 09 were comparatively higher than those for other the isolates. Interestingly, the most virulent isolate, Qin 08 (*M. robertsii*) exhibited a high amount of in vitro conidiation and a high growth rate. These two important biological attributes, and particularly conidiation, might affect horizontal transmission and disease development (Castrillo et al. 2011). In fact, conidial production can incite epizootics in host populations under suitable environmental conditions (Sun et al. 2002). This capability represents an advantage over other potential biological agents when considering use of fungal isolates for inoculative introduction. Moreover, during the bioassay experiment, one mealworm larva molted to pupae but was infected with the conidia of the most virulent isolate, Qin 08 (Figs. 8(a7, a8)). Notably, there is a specific entomopathogenic fungus (*Entomophaga maimaiga*) which has been introduced as a natural biocontrol of the gypsy moth, and several studies are focused on the pathogenicity of *E. maimaiga* against this harmful insect forest pest [for a review, see Hajek (1999)]. Here, we evaluated the pathogenicity of our screened isolate (Qin 08, *M. robertsii*) against the gypsy moth to consider an alternative approach for control of gypsy moths in forests (Fig. 8(B)).

As another point of interest, compared with other *Metarhizum* spp., the Qin 08 strain produced not only gold-pigmented exudate droplets on PDAY medium but also unpigmented droplets on the cadaver of mealworm larvae (Fig. 8(a5, a6)) and a dark-pigmented exudate on gypsy moth larvae (Fig. 8(b6)). Subsequently, we used the exudate to feed (2 μl per day for 1 week) the gypsy moth larvae in order to test the pathogenicity of these droplets against larvae. No biological activity or entomopathogenicity on larvae was noted and none of the larvae were infected. Hutwimmer et al. (2010) found that this exudate was composed of different toxic compounds such as destruxins that were toxic to insect pests. Possibly, the insecticide's active ingredients were not produced in a sufficient amount and/or the 2 μl exudate droplet was not enough to kill insect larvae in this study. Traditionally, fungal species have been separated based on the form, size, and color of their colony. Not surprisingly, our data suggest that morphological characterization was not an effective tool to delimitate *Metarhizium* isolates to species level in *M. anisopliae* s.l. particularly in the PARB clade as a result of the limited number and/or distinguishable phenotypic characters. This may explain why *M. anisopliae* s.l. is comprised of nine cryptic species that are consistent with other observations (Bidochka et al. 2005; Bischoff et al. 2009; Carrillo-Benítez et al. 2013). The integration of molecular phylogeny and morphological characterizations has greatly expedited screening of the isolates that belong to different species complexes. For instance, the bright green colony color and conidial dimension of the *M. flavoviride* species complex is the main distinguishing morphological feature relative to the *M. anisopliae* complex. In addition, the *M. flavoviride* species complex is even phylogenetically separated from the *M. anisopliae* complex (Keyser et al. 2015). However, this integration of morphology appears not to be a feature to delimitate phylogenetically closely related species in the PARB clade; it is difficult to obtain a provisional identification of isolates as to species-level classification.

Temperature is one of the key growth-limiting factors for any microorganism and can influence the success of biological control efforts. Here, we identified all *Metarhizium* isolates for their ability to grow across a gradient of cold conditions ranging from 27 to 5 °C. This cold activity experiment resulted in two findings. First, high variability in conidial cold activity was found among the *Metarhizium* spp. isolates after exposure to 10 °C for 15 days. Second, with two exceptions (Qin 04: 86.55% and Qin 18: 45.69%), the remaining of *Metarhizium* spp. isolates possess low germination rates at low temperature (5 °C). *Metarhizium* is intrinsically mesophilic. Some reports have verified the germination ability and virulence of some *Metarhizium* isolates at 5 °C that are indicative of a cold response transcriptional program mechanism (Bidochka et al. 2001; Fernandes et al. 2008). *Metarhizium* isolates from high elevation (Qin 01, Qin 02, and Qin 18) exposed to cold conditions (5 °C) are thought to exhibit an increased likelihood of conidial germination. Contrary to our expectation, Qin 04 isolated from low-elevation sites germinated at high percentages at low temperatures but Qin 01 and Qin 02 both isolated from high elevations tended to be cold-sensitive growth isolates with low levels of germinated conidia. This tenacity to cold is expected for *Metarhizium* because this ability is regulated by a cold shock response mechanism (CRP 1, CRP2) that evolutionarily was acquired by horizontal gene transfer from soil-dwelling bacteria within the same ecological niche (Fang and St Leger 2010). Cold activity can be associated with the environment from which the fungus was isolated (Santos et al. 2011). Here, Qin 04 might have resulted from the dispersal of fungal propagules from other elevations, both high and low, by environmental factors such as wind, host insects (Ormond et al. 2010), human activity, or other mechanisms. It could also be hypothesized that natural and/or anthropogenic long-distance dispersals are driving the abundance and diversity of fungi (Rouxel et al. 2011). Other factors besides translocation could be involved, such as phenotypic plasticity and cross protection in response to stress and environmental growth conditions (Rangel et al. 2015). This implies that, in a restricted number of cases, Qin 04 was involved in the responses and

adaptations to specific conditions that give rise to tolerance to cold. Our data suggest a non-significant relationship between *Metarhizium* spp. isolate distribution and particular climatic properties, in particular cold conditions. In conclusion, our molecular data seems to indicate that the intergenic locus, *Mz_IGS3*, distinguished indigenous *Metarhizium* species in the PARB clade with a significant bootstrap in comparison with 5́-*TEF*. Particularly, the reliability of amplification and high phylogenetic information yield of *Mz_IGS3* suggest this locus is a fine marker to delimitate unknown isolates of the *M. anisopliae* complex to species level (Kepler and Rehner 2013). Besides the phylogenetic analysis, we also evaluated some important features such as pathogenicity, sporulation, growth rate, and cold tolerance as environmental factors that may restrict the use of these insect pathogenic fungi for insect biological control. In our study, Qin 08, *M. robertsii*, possesses high pathogenicity, in vitro conidiation, and growth rate but it was notoriously susceptible to cold in comparison to the considerably higher tolerances of the Qin 04 and Qin 18 isolates. Obviously, selecting a thoroughly environmentally competent isolate to meet all important physiological requirements and/or traits can be a challenge to using insect pathogenic fungi as a biocontrol agent. In forest ecosystems, biodiversity is often higher than in other terrestrial biomes and forests are threatened by human influences such as degradation, fragmentation and loss of habitats, climate change, and pollution. We believe that any implemented sustainable management protocols should consider combining insect pathogenic fungi with other biological control agents as a judicious choice to managing invading insect populations, taking into consideration the forest ecosystem and biodiversity conservation. The knowledge gap about insect pathogenic fungi diversity, particularly of *Metarhizium* in forest regions in China, is of high concern and should be regarded as a research priority. The authors are explicitly concerned with *Metarhizium* distribution patterns in forest biomes, which require further study with specific research needed in different forest types and geographical locations in China.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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

References

Bidochka MJ, Kasperski JE, Wild GA (1998) Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. Can J Bot 76(7): 1198–1204

Bidochka MJ, Kamp AM, Lavender TM, Dekoning J, De Croos JNA (2001) Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? Appl Environ Microbiol 67(3):1335–1342. https://doi.org/10.1128/AEM.67.3.1335-1342.2001

Bidochka MJ, Menzies FV, Kamp AM (2002) Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. Arch Microbiol 178(6): 531–537. https://doi.org/10.1007/s00203-002-0490-7

Bidochka MJ, Small CLN, Spironello M (2005) Recombination within sympatric cryptic species of the insect pathogenic fungus *Metarhizium anisopliae*. Environ Microbiol 7(9):1361–1368. https://doi.org/10.1111/j.1462-5822.2005.00823.x

Bischoff JF, Rehner SA, Humber RA (2009) A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101(4):512–530. https://doi.org/10.3852/07-202

Braga GUL, Flint SD, Miller CD, Anderson AJ, Roberts DW (2001) Variability in response to UV-B among species and strains of *Metarhizium* isolated from sites at latitudes from 61°N to 54°S. J Invertebr Pathol 78(2):98–108. https://doi.org/10.1006/jipa.2001.5048

Carrillo-Benítez MG, Guzmán-Franco AW, Alatorre-Rosas R, Enríquez-Vara JN (2013) Diversity and genetic population structure of fungal pathogens infecting white grub larvae in agricultural soils. Microb Ecol 65(2):437–449. https://doi.org/10.1007/s00248-012-0124-9

Castrillo LA, Griggs MH, Ranger CM, Reding ME, Vandenberg JD (2011) Virulence of commercial strains of *Beauveria bassiana* and *Metarhizium brunneum* (*Ascomycota: Hypocreales*) against adult *Xylosandrus germanus* (*Coleoptera: Curculionidae*) and impact on brood. Biol Control 58(2):121–126. https://doi.org/10.1016/j.biocontrol.2011.04.010

Chandler D, Hay D, Reid AP (1997) Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. Appl Soil Ecol 5(2):133–141. https://doi.org/10.1016/S0929-1393(96)00144-8

Chen Z-H, Xu L, Yang F-l, Ji G-H, Yang J, Wang J-Y (2014) Efficacy of *Metarhizium anisopliae* isolate MAX-2 from Shangri-la, China under desiccation stress. BMC Microbiol 14(1):1

Choi Y-W, Hyde KD, Ho W (1999) Single spore isolation of fungi. Fungal Divers 3:29–38

de Faria MR, Wraight SP (2007) Mycoinsecticides and Mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol Control 43(3):237–256. https://doi.org/10.1016/j.biocontrol.2007.08.001

Driver F, Milner RJ, Trueman JW (2000) A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycol Res 104(2):134–150. https://doi.org/10.1017/S0953756299001756

Du X-C, Ren Y, Dang G-D, Lundholm J (2011) Distribution and plant community associations of the understory bamboo *Fargesia*

qinlingensis in the Foping National Nature Reserve, China. Ann For Sci 68(7):1197–1206. https://doi.org/10.1007/s13595-011-0104-0

Fang W, St Leger RJ (2010) RNA binding proteins mediate the ability of a fungus to adapt to the cold. Environ Microbiol 12(3):810–820. https://doi.org/10.1111/j.1462-2920.2009.02127.x

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17(6):368–376. https://doi.org/10.1007/BF01734359

Fernandes ÉKK, Rangel DEN, Moraes ÁML, Bittencourt VREP, Roberts DW (2008) Cold activity of *Beauveria* and *Metarhizium*, and thermotolerance of *Beauveria*. J Invertebr Pathol 98(1):69–78. https://doi.org/10.1016/j.jip.2007.10.011

Fernandes ÉKK, Keyser CA, Chong JP, Rangel DEN, Miller MP, Roberts DW (2010) Characterization of *Metarhizium* species and varieties based on molecular analysis, heat tolerance and cold activity. J Appl Microbiol 108(1):115–128. https://doi.org/10.1111/j.1365-2672.2009.04422.x

Gao Q, Jin K, Ying S-H, Zhang Y, Xiao G, Shang Y, Duan Z, Hu X, Xie X-Q, Zhou G, Peng G, Luo Z, Huang W, Wang B, Fang W, Wang S, Zhong Y, Ma L-J, St Leger RJ, Zhao G-P, Pei Y, Feng M-G, Xia Y, Wang C (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 7(1):e1001264. https://doi.org/10.1371/journal.pgen.1001264

Garrido-Jurado I, Fernández-Bravo M, Campos C, Quesada-Moraga E (2015) Diversity of entomopathogenic *Hypocreales* in soil and phylloplanes of five Mediterranean cropping systems. J Invertebr Pathol 130:97–106. https://doi.org/10.1016/j.jip.2015.06.001

Glare TR, Reay SD, Nelson TL, Moore R (2008) *Beauveria caledonica* is a naturally occurring pathogen of forest beetles. Mycol Res 112(3):352–360. https://doi.org/10.1016/j.mycres.2007.10.015

Hajek AE (1999) Pathology and epizootiology of *Entomophaga maimaiga* infections in forest *Lepidoptera*. Microbiol Mol Biol Rev 63(4):814–835

Hallsworth JE, Magan N (1996) Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules. Appl Environ Microbiol 62(7):2435–2442

Han SK, Lee D, Lee H, Kim D, Son HG, Yang J-S, Lee S-JV, Kim S (2016) OASIS 2: online application for survival analysis 2 with features for the analysis of maximal lifespan and healthspan in aging research. Oncotarget 7(35):56147–56152. 10.18632/oncotarget.11269

Hutwimmer S, Wang H, Strasser H, Burgstaller W (2010) Formation of exudate droplets by *Metarhizium anisopliae* and the presence of destruxins. Mycologia 102(1):1–10. https://doi.org/10.3852/09-079

Inglis G, Johnson D, Goettel M (1997) Field and laboratory evaluation of two conidial batches of *Beauveria bassiana* (Balsamo) Vuillemin against grasshoppers. Can Entomol 129(01):171–186. https://doi.org/10.4039/Ent129171-1

Ivey FD, Hodge PN, Turner GE, Borkovich KA (1996) The G alpha i homologue *gna-1* controls multiple differentiation pathways in *Neurospora crassa*. Mol Biol Cell 7(8):1283–1297. https://doi.org/10.1091/mbc.7.8.1283

Johnson D, Sung G-H, Hywel-Jones NL, Luangsa-Ard JJ, Bischoff JF, Kepler RM, Spatafora JW (2009) Systematics and evolution of the genus *Torrubiella* (*Hypocreales, Ascomycota*). Mycol Res 113(3):279–289. https://doi.org/10.1016/j.mycres.2008.09.008

Kamp AM, Bidochka MJ (2002) Conidium production by insect pathogenic fungi on commercially available agars. Lett Appl Microbiol 35(1):74–77. https://doi.org/10.1046/j.1472-765X.2002.01128.x

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30(4):772–780. https://doi.org/10.1093/molbev/mst010

Kepler RM, Rehner SA (2013) Genome-assisted development of nuclear intergenic sequence markers for entomopathogenic fungi of the *Metarhizium anisopliae* species complex. Mol Ecol 13(2):210–217. https://doi.org/10.1111/1755-0998.12058

Kepler RM, Humber RA, Bischoff JF, Rehner SA (2014) Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. Mycologia 106(4):811–829. https://doi.org/10.3852/13-319

Kepler RM, Ugine TA, Maul JE, Cavigelli MA, Rehner SA (2015) Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ Microbiol 17(8):2791–2804. https://doi.org/10.1111/1462-2920.12778

Kessler P, Matzke H, Keller S (2003) The effect of application time and soil factors on the occurrence of *Beauveria brongniartii* applied as a biological control agent in soil. J Invertebr Pathol 84(1):15–23. https://doi.org/10.1016/j.jip.2003.08.003

Keyser CA, De Fine Licht HH, Steinwender BM, Meyling NV (2015) Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. BMC Microbiol 15(1):249. https://doi.org/10.1186/s12866-015-0589-z

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16(2):111–120. https://doi.org/10.1007/BF01731581

Lopes RB, Mesquita ALM, Tigano MS, Souza DA, Martins I, Faria M (2013a) Diversity of indigenous *Beauveria* and *Metarhizium* spp. in a commercial banana field and their virulence toward *Cosmopolites sordidus* (*Coleoptera: Curculionidae*). Fungal Ecol 6(5):356–364. https://doi.org/10.1016/j.funeco.2013.06.007

Lopes RB, Souza DA, Oliveira CM, Faria M (2013b) Genetic diversity and pathogenicity of *Metarhizium* spp. associated with the white grub *Phyllophaga capillata* (Blanchard) (*Coleoptera: Melolonthidae*) in a soybean field. Neotrop Entomol 42(4):436–438. https://doi.org/10.1007/s13744-013-0146-0

Luan F, Zhang S, Wang B, Huang B, Li Z (2013) Genetic diversity of the fungal pathogen *Metarhizium* spp., causing epizootics in Chinese burrower bugs in the Jingting Mountains, eastern China. Mol Biol Rep 40(1):515–523. https://doi.org/10.1007/s11033-012-2088-8

Maddison WP, Maddison DR (2015) Mesquite: a modular system for evolutionary analysis. Version 3.04

Medo J, Cagáň Ľ (2011) Factors affecting the occurrence of entomopathogenic fungi in soils of Slovakia as revealed using two methods. Biol Control 59(2):200–208. https://doi.org/10.1016/j.biocontrol.2011.07.020

Meyling NV, Eilenberg J (2006) Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. Agric Ecosyst Environ 113(1–4):336–341. https://doi.org/10.1016/j.agee.2005.10.011

Meyling NV, Eilenberg J (2007) Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. Biol Control 43(2):145–155. https://doi.org/10.1016/j.biocontrol.2007.07.007

Meyling NV, LuBeck M, Buckley EP, Eilenberg J, Rehner SA (2009) Community composition, host range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and seminatural habitats. Mol Ecol 18(6):1282–1293. https://doi.org/10.1111/j.1365-294X.2009.04095.x

Meyling NV, Thorup-Kristensen K, Eilenberg J (2011) Below- and aboveground abundance and distribution of fungal entomopathogens in experimental conventional and organic cropping systems. Biol Control 59(2):180–186. https://doi.org/10.1016/j.biocontrol.2011.07.017

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Gateway Computing Environments Workshop (GCE). IEEE, New Orleans, pp 1–8

Milne I, Lindner D, Bayer M, Husmeier D, McGuire G, Marshall DF, Wright F (2009) TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multicore desktops. Bioinformatics 25(1):126–127. https://doi.org/10.1093/bioinformatics/btn575

Muñiz-Reyes E, Guzmán-Franco AW, Sánchez-Escudero J, Nieto-Angel R (2014) Occurrence of entomopathogenic fungi in tejocote (*Crataegus mexicana*) orchard soils and their pathogenicity against *Rhagoletis pomonella*. J Appl Microbiol 117(5):1450–1462. https://doi.org/10.1111/jam.12617

Nishi O, Hasegawa K, Iiyama K, Yasunaga-Aoki C, Shimizu S (2011) Phylogenetic analysis of *Metarhizium* spp. isolated from soil in Japan. Appl Entomol Zool 46(3):301–309. https://doi.org/10.1007/s13355-011-0045-y

Nishi O, Iiyama K, Yasunaga-Aoki C, Shimizu S (2015) Phylogenetic status and pathogenicity of *Metarhizium majus* isolated from a fruit beetle larva in Japan. Mycol Prog 14(8):58. https://doi.org/10.1007/s11557-015-1082-7

Nunes Rocha LF, Hua Tai MH, dos Santos AH, dos Santos Albernaz DA, Humber RA, Luz C (2009) Occurrence of invertebrate-pathogenic fungi in a Cerrado ecosystem in Central Brazil. Biocontrol Sci Tech 19(5–6):547–553. https://doi.org/10.1080/09583150902789337

Ormond EL, Thomas APM, Pugh PJA, Pell JK, Roy HE (2010) A fungal pathogen in time and space: the population dynamics of *Beauveria bassiana* in a conifer forest. FEMS Microbiol Ecol 74(1):146–154. https://doi.org/10.1111/j.1574-6941.2010.00939.x

Pérez-González VH, Guzmán-Franco AW, Alatorre-Rosas R, Hernández-López J, Hernández-López A, Carrillo-Benítez MG, Baverstock J (2014) Specific diversity of the entomopathogenic fungi *Beauveria* and *Metarhizium* in Mexican agricultural soils. J Invertebr Pathol 119:54–61. https://doi.org/10.1016/j.jip.2014.04.004

Popowska-Nowak E, Skrzecz I, Tumialis D, Pezowics E, Samborska I, Goral K (2016) Entomopathogenic fungi in the soils of forest plantations: towards the control of large pine weevil, *Hylobius abietis*. Balt For 22(1):8–15

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14(9):817–818. https://doi.org/10.1093/bioinformatics/14.9.817

Posadas JB, Comerio RM, Mini JI, Nussenbaum AL, Lecuona RE (2012) A novel dodine-free selective medium based on the use of cetyl trimethyl ammonium bromide (CTAB) to isolate *Beauveria bassiana*, *Metarhizium anisopliae* sensu lato and *Paecilomyces lilacinus* from soil. Mycologia 104(4):974–980. https://doi.org/10.3852/11-234

Qian H, Ricklefs RE (2016) Out of the tropical lowlands: latitude versus elevation. Trends Ecol Evol 31(10):738–741. https://doi.org/10.1016/j.tree.2016.07.012

Rangel DEN, Braga GUL, Fernandes ÉKK, Keyser CA, Hallsworth JE, Roberts DW (2015) Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. Curr Genet 61(3):383–404. https://doi.org/10.1007/s00294-015-0477-y

Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear *ITS* and *EF1-α* sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97(1):84–98

Rehner SA, Posada F, Buckley EP, Infante F, Castillo A, Vega FE (2006) Phylogenetic origins of African and Neotropical *Beauveria bassiana* s.l. pathogens of the coffee berry borer, *Hypothenemus hampei*. J Invertebr Pathol 93(1):11–21. https://doi.org/10.1016/j.jip.2006.04.005

Rehner SA, Minnis AM, Sung G-H, Luangsa-ard JJ, Devotto L, Humber RA (2011) Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. Mycologia 103(5):1055–1073. https://doi.org/10.3852/10-302

Reich PB, Oleksyn J, Modrzynski J, Mrozinski P, Hobbie SE, Eissenstat DM, Chorover J, Chadwick OA, Hale CM, Tjoelker MG (2005) Linking litter calcium, earthworms and soil properties: a common garden test with 14 tree species. Ecol Lett 8(8):811–818. https://doi.org/10.1111/j.1461-0248.2005.00779.x

Rezende JM, Zanardo ABR, da Silva Lopes M, Delalibera I, Rehner SA (2015) Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. BioControl 60(4):495–505. https://doi.org/10.1007/s10526-015-9656-5

Rocha LFN, Inglis PW, Humber RA, Kipnis A, Luz C (2013) Occurrence of *Metarhizium* spp. in Central Brazilian soils. J Basic Microbiol 53(3):251–259. https://doi.org/10.1002/jobm.201100482

Rouxel T, Grandaubert J, Hane JK, Hoede C, van de Wouw AP, Couloux A, Dominguez V, Anthouard V, Bally P, Bourras S, Cozijnsen AJ, Ciuffetti LM, Degrave A, Dilmaghani A, Duret L, Fudal I, Goodwin SB, Gout L, Glaser N, Linglin J, Kema GHJ, Lapalu N, Lawrence CB, May K, Meyer M, Ollivier B, Poulain J, Schoch CL, Simon A, Spatafora JW, Stachowiak A, Turgeon BG, Tyler BM, Vincent D, Weissenbach J, Amselem J, Quesneville H, Oliver RP, Wincker P, Balesdent M-H, Howlett BJ (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by repeat-induced point mutations. Nat Commun 2:202. https://doi.org/10.1038/ncomms1189

Santos MP, Dias LP, Ferreira PC, Pasin LAAP, Rangel DEN (2011) Cold activity and tolerance of the entomopathogenic fungus *Tolypocladium* spp. to UV-B irradiation and heat. J Invertebr Pathol 108(3):209–213. https://doi.org/10.1016/j.jip.2011.09.001

Spatafora JW, Sung GH, Sung JM, Hywel-Jones NL, White JF (2007) Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. Mol Ecol 16(8):1701–1711. https://doi.org/10.1111/j.1365-294X.2007.03225.x

Sun J, Fuxa JR, Henderson G (2002) Sporulation of *Metarhizium anisopliae* and *Beauveria bassiana* on *Coptotermes formosanus* and in vitro. J Invertebr Pathol 81(2):78–85. https://doi.org/10.1016/S0022-2011(02)00152-0

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30(12):2725–2729. https://doi.org/10.1093/molbev/mst197

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31(1):21–32. https://doi.org/10.1006/fgbi.2000.1228

Vanninen I (1996) Distribution and occurrence of four entomopathogenic fungi in Finland: effect of geographical location, habitat type and soil type. Mycol Res 100(1):93–101. https://doi.org/10.1016/S0953-7562(96)80106-7

Wang Y-C, Wang D-X, Liu H-R (2013) Altitudinal distribution of seed plants in Foping and Changqing, Qinling Mountains, China. Ann Bot Fenn 50(1–2):43–49. https://doi.org/10.5735/085.050.0106

Wyrebek M, Huber C, Sasan RK, Bidochka MJ (2011) Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. Microbiology 157(10):2904–2911. https://doi.org/10.1099/mic.0.051102-0