Species Diversity and Virulence Potential of the *Beauveria bassiana* Complex and *Beauveria scarabaeidicola* Complex

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**INTRODUCTION**

*Beauveria* is a very important fungal resource, with some species having great economic and ecological value (Zimmermann, 2007; Rehner et al., 2011; Wang Y. et al., 2020). *Beauveria bassiana* (Bals.-Criv.) Vuill. and *B. brongniartii* (Sacc.) Petch are well-known environmentally safe alternatives to using chemical pesticides to control agricultural pests (Zimmermann, 2007; Rehner et al., 2011). *Beauveria pseudobassiana* S.A. Rehner and Humber has also been shown to have great potential in the biocontrol of numerous insect pests (Wang Y. et al., 2020). The entomopathogenic fungi *Beauveria* spp. are a class of environmentally friendly fungal pathogens that play an important role in controlling insect populations in nature (Luo et al., 2018; McKinnon et al., 2018). Some *Beauveria* species, as endophytes or soil and rhizosphere inhabitants, have been considered for potential use as biocontrol agents against plant pathogens by concerned practitioners, such as agriculturalists and plant pathologists. These species can produce an array of bioactive metabolites that limit the growth of some fungal plant pathogens and induce plant systemic resistance against the pathogenic bacterium (Ownley et al., 2010).
Beauveria bassiana is the most widely used fungus available commercially for controlling agricultural and forestry pests (Li et al., 2011). Products based on this species have been developed in many countries around the world (Goettel et al., 2005; Faria and Wraight, 2007; Li et al., 2011). However, a growing body of molecular evidence has demonstrated that B. bassiana, originally known as a generalist with a global distribution, encompasses cryptic lineages adapted to specific hosts or ecologies (Li et al., 2011; Rehner et al., 2011). Many initially identified B. bassiana isolates may belong to any of the species in the B. bassiana complex, such as B. rudraprayagi Y. Agrawal, Mual and Shenoy, B. staphylinidicola (Kobayasi and Shimizu) B. Shrestha, Kepler and Spatafora, and B. peruviensis D.E. Bustamante, M.S. Calderon, M. Oliva, and S. Leiva (Rehner et al., 2011; Agrawal et al., 2014; Kepler et al., 2017; Bustamante et al., 2019). Therefore, the abovementioned mycoinsecticide formulations of B. bassiana are not likely all based on B. bassiana.

Beauveria scarabaeidicola (Kobayasi) S.A. Rehner and Kepler is widely distributed in Oceania and Asia and named after its host adult beetle (Coleoptera: Scarabaeidae). It was originally described as Cordyceps scarabaeicola occurring in its sexual morph on an adult scarab beetle in New Guinea (Kobayasi and Shimizu, 1976). Cordyceps scarabaeicola has also been reported occasionally from many Asian countries, including China, Japan, and Korea (Shrestha et al., 2014). In an important phylogenetic study of Beauveria, a new entomopathogenic species, B. sungii S.A. Rehner and R.A. Humber, was described as a scarab-killing pathogen (hosts of all B. sungii isolates were identified as scarabs) (Rehner et al., 2011). Later, however, Shrestha et al. (2014) demonstrated that the teleomorphic stage of B. sungii was C. scarabaeicola based on morphological and phylogenetic evidence. Because C. scarabaeicola was described earlier than B. sungii, Kepler et al. (2017) recommended B. scarabaeicola as the name of this species. Recently, Chen et al. (2019) proposed a new species, B. yunnanensis, a Chinese species parasitic on Lepidoptera pupa buried in soil that was a sister lineage to B. scarabaeicola.

During surveys of entomopathogenic fungi from different regions in Yunnan Province, China, and Chiang Rai Province, Thailand, over the past 4 years, approximately 15 Beauveria spp. were found and identified (Table 1). In this study, we aimed to: (1) reveal the hidden species diversity of the B. bassiana complex and B. scarabaeidicola complex based on phylogenetic analyses and morphological observation and (2) assess the biocontrol potential of species in the B. bassiana complex and B. scarabaeidicola complex through pathological tests on the lepidopteran Bombyx mori and the coleopteran Tenebrio molitor larvae as well as Protaetia brevitarsis adults.

MATERIALS AND METHODS

Soil and Specimen Collection

All the soil samples and the majority of Beauveria specimens were collected from Yunnan Province in China. Some specimens were collected from Chiang Rai Province in Thailand. Soil samples and specimens were noted and photographed in the fields, and then carefully placed in plastic containers at low temperature. Afterward, they were carried to the laboratory and stored at 4°C before examination and isolation.

Fungal Isolation and Culture

Beauveria strains were isolated from the soil samples using the Tenebrio molitor baiting method (Keyser et al., 2015). Conidia developing on insect cadavers were transplanted onto plates of potato dextrose agar (PDA; potato 200 g/L, dextrose 20 g/L, agar 20 g/L) and cultured at 25°C. Teleomorph specimens were rinsed with tap water, washed with sterile distilled water, and then dried on sterile filter paper. To obtain axenic cultures, white tissue inside the sclerotia of the teleomorph specimens was removed and inoculated onto PDA plates using a sterilized dissecting knife. Colonies of the isolated filamentous fungi appearing in the culture were transferred onto fresh PDA media. The purified fungal strains were maintained in a culture room at 25°C or transferred to PDA slants and stored at 4°C. Specimens were deposited in the Yunnan Herbal Herbarium (YHH) at the Institute of Herb Biotic Resources of Yunnan University. Cultures were stored in the Yunnan Fungal Culture Collection (YFCC) at the Institute of Herb Biotic Resources of Yunnan University.

Morphological Observations

Specimens were examined using an Olympus SZ61 stereomicroscope (Olympus Corporation, Tokyo, Japan). Cultures on PDA slants were transferred to PDA plates and then incubated at 25°C for 14 days. For morphological evaluation, microscope slides were prepared by placing mycelia from the cultures on PDA medium blocks (5 mm diameter) and then overlaid with a coverslip. Medan dye solution was used to observe asci and ascospores. Other structures were mounted in water. Micro-morphological observations and measurements were performed using a light microscope (CX40, Olympus Corporation, Tokyo, Japan) and a scanning electron microscope (Quanta 200 FEG, FEI Company, Hillsboro, United States). Length to width ratios are given as Q. Mean values for length, width, and Q are indicated by L\textsuperscript{m}, W\textsuperscript{m}, and Q\textsuperscript{m}, respectively.

DNA Extraction, PCR and Sequencing

Specimens and axenic living cultures were prepared for DNA extraction. Genomic DNA was extracted using the Genomic DNA Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The primer pair nrSSU-CoF and nrSSU-CoR was used to amplify a fraction of the nuclear ribosomal small subunit (nrSSU) (Wang et al., 2015). Primer pair L5R and LR0R (Vilgalys and Hester, 1990; Rehner and Samuels, 1994) was used to amplify a fraction of the nuclear ribosomal large subunit (nrLSU) and EF1α-EF and EF1α-ER (Bischoff et al., 2006; Sung et al., 2007) for the translation elongation factor 1α (TEF). For amplification of the largest and second largest subunits of the RNA polymerase II (RPB1 and RPB2), PCR primer pairs RPB1-5′F/RPB1-5′R and RPB2-5′F/RPB2-5′R (Bischoff et al., 2006; Sung et al., 2007).
TABLE 1 | Specimen information and GenBank accession numbers for sequences used in this study.

| Taxon                     | Voucher information | Host/substrate | GenBank accession number | References |
|---------------------------|---------------------|----------------|--------------------------|------------|
| Beaviera asiatica     | ARSEF 2641T         | Hymenoptera: Formicidae | AY531917 HQ880880 HQ880952 HQ880739 | Rehner et al., 2011 |
| Beaviera araneola     | GZAC 15031T         | Araneae        | KT961699 KT961701 KT961698 | Chen et al., 2017 |
| Beaviera asiatica     | ARSEF 4850T         | Coleoptera: Cerambycidae | AY531937 HQ880859 HQ880931 HQ880718 | Rehner et al., 2011 |
| Beaviera asiatica     | YFCC 5600           | Coleoptera: Cerambycidae | MN576996 MN576886 MN576940 MW168177 | Wang Y. B. et al., 2020; This study |
| Beaviera australis     | ARSEF 4598T         | Soil           | HQ880995 HQ880861 HQ880933 HQ880720 | Rehner et al., 2011 |
| Beaviera baoshanensis | CCTC 2018011T       | Coleoptera: Chrysomelidae | MG642897 MG642854 MG642867 | Chen et al., 2019 |
| Beaviera bassiana     | ARSEF 1564T         | Lepidoptera: Arctiidae | HQ880974 HQ880833 HQ880905 HQ880692 | Rehner et al., 2011 |
| Beaviera bassiana     | ARSEF 7518           | Coleoptera: Pamphiliidae | HQ880975 HQ880834 HQ880906 HQ880693 | Rehner et al., 2011 |
| Beaviera bassiana     | YFCC 3369           | Coleoptera: Scarabaeidae | MN576994 MN576884 MN576938 MW168176 | Wang Y. B. et al., 2020; This study |
| Beaviera blattidicola | MCA 1727T           | Blattodea: Blattidae | MF416483 MF416640 | Kepler et al., 2017 |
| Beaviera brongniartii | ARSEF 617T          | Coleoptera: Scarabaeidae | HQ880991 HQ880854 HQ880926 HQ880713 | Rehner et al., 2011 |
| Beaviera brongniartii | YFCC 3240           | Coleoptera: Scarabaeidae | MN576995 MN576885 MN576939 MW168175 | Wang Y. B. et al., 2020; This study |
| Beaviera caledonica   | ARSEF 2567T         | Soil           | EF469057 HQ880889 HQ880961 HQ880748 | Rehner et al., 2011 |
| Beaviera caledonica   | YFCC 7025           | Coleoptera: Cerambycidae | MN576997 MN576887 MN576941 MW168178 | Wang Y. B. et al., 2020; This study |
| Beaviera diapheromeniphila | QCNE 186272T | Phasmatodea: Diapheromeridae | JQ968610 JX003848 | Sanjuan et al., 2014 |
| Beaviera diapheromeniphila | QCNE 186714 | Phasmatodea: Diapheromeridae | MF416491 MF416648 | Kepler et al., 2017 |
| Beaviera hoplocheli    | Bt116          | Coleoptera: Melolonthidae | KC339703 KM453965 KM453966 KM453967 | Robène-Soustrade et al., 2015 |
| Beaviera hoplocheli    | MNHN-RF-06107T     | Coleoptera: Melolonthidae | KC339702 KM453965 KM453963 KM453971 | Robène-Soustrade et al., 2015 |
| Beaviera kipukae      | ARSEF 7032T         | Homoptera: Delphacidae | HQ881005 HQ880875 HQ880947 HQ880734 | Rehner et al., 2011 |
| Beaviera lili         | ARSEF 11741T        | Coleoptera: Coccinellidae | JN689371 JN689374 JN689370 JN689373 | Zhang et al., 2012 |
| Beaviera locustiphila | TSS81              | Orthoptera: Romaleidae | JQ968619 JX003847 JX003845 | Sanjuan et al., 2014 |
| Beaviera majiangensis | GZAC 121241T         | Coleoptera: Scarabaeida | MG052640 MG052644 MG052639 | Chen et al., 2018 |
| Beaviera majiangensis | YFCC 852            | Coleoptera: Scarabaeidae | MW168229 MW168195 MW168212 MW168179 | This study |
| Beaviera malawiensis    | ARSEF 7760T         | Coleoptera: Cerambycidae | DG376246 HQ880897 HQ880969 HQ880756 | Rehner et al., 2011 |
| Beaviera malawiensis    | YFCC 853            | Coleoptera: Scarabaeidae | MW168230 MW168196 MW168213 MW168180 | This study |

(Continued)
| Taxon                    | Voucher information | Host/substrate                  | GenBank accession number | References                  |
|-------------------------|---------------------|---------------------------------|--------------------------|-----------------------------|
|                         |                     |                                 | TEF | RPB1 | RPB2 | Bloc |                       |
| Beauveria medogensis    | 2898                | Soil                            | KU994833                  | KU994835 | KU994834 | KU994836 | Imoulan et al., 2016 |
| Beauveria medogensis    | YFCC 854            | Coleopteran adult               | MW168231                  | MW168197 | MW168214 | MW168181 | This study           |
| Beauveria peruviensis   | UTRP19 = ARSEF 14196T | Coleoptera: Curculionidae       | MN094781                  | MN100118 | MN094757 |         | Bustamante et al., 2019 |
| Beauveria peruviensis   | UTRF35              | Coleoptera: Curculionidae       | MN094771                  | MN100115 | MN094755 |         | Bustamante et al., 2019 |
| Beauveria polyrhachicola| YFCC 859T           | Hymenoptera: Formicidae         | MW168236                  | MW168202 | MW168219 | MW168184 | This study           |
| Beauveria polyrhachicola| YFCC 867            | Hymenoptera: Formicidae         | OM373098                  | OM373099 | OM304364 | OM373100 | This study           |
| Beauveria pseudobassiana| ARSEF 3405T         | Lepidoptera: Tortricidae        | AY531931                  | HQ880864 | HQ880936 | HQ880723 | Rehner et al., 2011 |
| Beauveria pseudobassiana| YFCC 1806007        | Coleoptera: Scarabaeidae        | MN523553                  | MN523582 | MN523611 | MN862889 | Wang Y. et al., 2020 |
| Beauveria rudraprayagi  | MTCC 8017T          | Lepidoptera: Bombycidae         | JC990914                  | JC990892 | JC990870 | JC990848 | Agrawal et al., 2014 |
| Beauveria scarabaeidica | ARSEF 1685          | Coleoptera: Scarabaeidae        | AY531899                  | HQ880881 | HQ880953 | HQ880740 | Rehner et al., 2011 |
| Beauveria scarabaeidica | ARSEF 5689          | Coleoptera: Scarabaeidae        | DQ522335                  | DQ522380 | DQ522431 | HQ880741 | Rehner et al., 2011 |
| Beauveria scarabaeidica | ARSEF 7043          | Coleoptera: Scarabaeidae        | AY531948                  | HQ880883 | HQ880955 | HQ880742 | Rehner et al., 2011 |
| Beauveria scarabaeidica | ARSEF 7279          | Coleoptera: Scarabaeidae        | HQ881009                  | HQ880885 | HQ880957 | HQ880744 | Rehner et al., 2011 |
| Beauveria scarabaeidica | ARSEF 7281          | Coleoptera: Scarabaeidae        | HQ881011                  | HQ880887 | HQ880959 | HQ880746 | Rehner et al., 2011 |
| Beauveria scarabaeidica | YFCC 865            | Coleoptera: Scarabaeidae        | MW168243                  | MW168209 | MW168226 | MW168191 | This study           |
| Beauveria sinensis      | BUB 504             | Orthoptera: Gryllidae           | MG642885                  | MG642852 | MG642865 |         | Chen et al., 2019    |
| Beauveria sinensis      | RCEF 3903T          | Lepidoptera: Geometridae        | HQ270151                  | JX524283 | JX524284 |         | Chen et al., 2013    |
| Beauveria songmingensis | YFCC 860T           | Coleoptera: Scarabaeidae        | MW168238                  | MW168204 | MW168221 | MW168186 | This study           |
| Beauveria songmingensis | YFCC 861            | Coleoptera: Scarabaeidae        | MW168239                  | MW168205 | MW168222 | MW168187 | This study           |
| Beauveria staphylindicola| ARSEF 5718          | Coleoptera: Staphylidae         | EF468776                  | EF468881 |         | AY883907 | Sung et al., 2007    |
| Beauveria staphylindicola| YFCC 855            | Coleoptera: Cerambycidae        | MW168232                  | MW168198 | MW168215 | MW168182 | This study           |
| Beauveria subscaranidica | YFCC 863T           | Coleoptera: Scarabaeidae        | MW168241                  | MW168207 | MW168224 | MW168189 | This study           |
| Beauveria subscaranidica| YFCC 864            | Coleoptera: Scarabaeidae        | MW168242                  | MW168208 | MW168225 | MW168190 | This study           |
| Beauveria varroae       | ARSEF 8257T         | Coleoptera: Curculionidae       | HQ881002                  | HQ880872 | HQ880944 | HQ880731 | Rehner et al., 2011 |
| Beauveria vermiconia    | ARSEF 2922T         | Soil                            | AY531920                  | HQ880894 | HQ880966 | HQ880753 | Rehner et al., 2011 |
| Beauveria yunnanensis   | CCTCC AF 2018010T   | Lepidoptera: popula             | MG642900                  | MG642857 | MG642870 |         | Chen et al., 2019    |
| Beauveria yunnanensis   | YFCC 3105           | Coleoptera: Scarabaeidae        | MN576999                  | MN576889 | MN576943 |         | Wang Y. B. et al., 2020 |
| Beauveria yunnanensis   | YFCC 862            | Coleoptera: Scarabaeidae        | MW168240                  | MW168206 | MW168223 | MW168188 | This study           |

Boldface: data generated in this study. ^T ex-type material.
were employed. The *Bloc* fragment was amplified using primer pair B5.1F/B3.1R (Rehner et al., 2006). All the PCR reactions were performed in a final volume of 50 µL and contained 25 µL of 2 × Taq PCR Master Mix (Tiangen Biotech Co. Ltd, Beijing, China), 0.5 µL of each primer (10 µM), 1 µL of genomic DNA, and 23 µL of RNase-free water. Target gene amplification and sequencing were performed according to the methods described in our previous study (Wang Y. B. et al., 2020).

### Phylogenetic Analyses

Phylogenetic analyses were based on six gene (nrSSU, nrLSU, TEF, RPB1, RPB2, and *Bloc*) sequences. The sequences were retrieved from GenBank and combined with those generated in our study. Taxon information and GenBank accession numbers were provided in Supplementary Table 1 and Table 1. Sequences were aligned using MAFFT v.7.1 After alignment, the sequences of the genes were concatenated. Conflicts among the six genes were not in conflict. The data partitions were defined for the combined dataset using PartitionFinder V1.1.1 (Lanfear et al., 2012). Phylogenetic analyses were conducted using BI and ML methods employing MrBayes v3.1.2 and RaxML 7.0.3, respectively (Ronquist and Huelsenbeck, 2003; Stamatakis et al., 2008). The BI analysis was run on MrBayes v3.1.2 for five million generations using a GTR+G+I model determined by jModelTest version 2.1.4 (Darriba et al., 2012). GTR+I was selected as the optimal model for ML analysis, and 1,000 rapid bootstrap replicates were performed on the dataset.

The first analysis based on the combined five-gene (nrSSU+nrLSU+TEF+RPB1+RPB2) dataset was performed using the following taxa: *Akanthomyces*, *Amphichorda*, *Ascopolyporus*, *Beauveria*, *Blackwollomyces*, *Cordyceps*, *Gibellula*, *Hevansia*, *Samsoniella*, and *Simplicillium*. Two taxa of *Trichoderma* were designated as outgroups. The second analysis based on the combined four-gene (TEF+RPB1+RPB2+*Bloc*) sequences was performed using *Beauveria* taxa.

We applied a (phylo-) genetic distance matrix calculation for the combined four-gene (TEF+RPB1+RPB2+*Bloc*) sequences to assess species boundaries in the *B. bassiana* complex and *B. scarabaeidicola* complex (Table 2). The pairwise genetic distances of most *Beauveria* lineages (Supplementary Table 2) were measured based on the Kimura 2-parameter model using MEGA6 software (Tamura et al., 2013).

### Conidial Viability of *Beauveria* spp. Isolates

A total of 19 *Beauveria* spp. isolates (Table 3) were analyzed for their conidial viability using the method described by Imoulan et al. (2011). The conidial viability of each isolate was confirmed by inoculating three tubes of 3 ml PDB media (potato 200 g/L, dextrose 20 g/L) with 0.1 ml of conidia suspension (3 × 10⁶ conidia/ml). Only isolates with conidial viability greater than 65% were tested for pathogenicity toward *B. mori*, *T. molitor*, and *P. brevitarsis*.

### Virulence Assay of *Beauveria* spp. Isolates

A total of 10 *Beauveria* spp. isolates from the *B. bassiana* complex and *B. scarabaeidicola* complex were tested for their pathogenicity to *B. mori* and *T. molitor* larvae in addition to *P. brevitarsis* adults. Conidia for each isolate were obtained from 4-week-old cultures grown on malt extract agar plates, suspended in a sterile aqueous solution of 0.01% Tween 80, and mixed vigorously until homogeneous conidial suspensions were produced. Quantification of the conidia was performed using a hemocytometer under a light microscope at 400 × magnification. All of the suspensions were adjusted to 1 × 10⁶ conidia/ml.

The tested insects were individually placed in sterilized rearing boxes and 10 µl of conidial suspension was applied to the surface of each insect. A diet was provided for each insect and renewed as needed. Control groups were treated with the same volume of a sterile aqueous solution of 0.01% Tween 80. The test was replicated three times with 50 insects per replicate. All of the test groups were kept at 25°C under a 12:12 h photoperiod cycle. The numbers of dead insects were recorded every 12 h for a 30 day period, which was used to determine the percentage of mortality. Cadavers were removed, immediately surface-disinfected, and individually placed and maintained in rearing box chambers. Mycelium samples from cadavers were aseptically removed and cultured on PDA for microscope examination, DNA extraction, and TEF sequencing to confirm that mortality was caused by the inoculated fungal strain.

### RESULTS

#### Sequencing and Phylogenetic Analyses

The combined five-gene dataset included sequences from 123 fungal taxa. The final dataset consisted of 5,001 bp of sequence data (nrSSU 1,138 bp, nrLSU 910 bp, TEF 1,047 bp, RPB1 781 bp, and RPB2 1,125 bp). Eleven well-supported clades were recognized based on both Bayesian inference (BI) and maximum likelihood (ML) analyses of the combined five-gene dataset of 123 taxa from Cordycipitaceae and *Trichoderma*, which accommodate species of the genera *Akanthomyces*, *Amphichorda*, *Ascopolyporus*, *Beauveria*, *Blackwollomyces*, *Cordyceps*, *Gibellula*, *Hevansia*, *Samsoniella*, *Simplicillium*, and *Trichoderma* (Supplementary Figure 1). The phylogenetic analyses also revealed the species diversity of the *B. bassiana* complex and *B. scarabaeidicola* complex in *Beauveria* clades. This suggested that the groups composed of the *B. bassiana* complex and *B. scarabaeidicola* complex should be genetically composed of at least four species (Supplementary Figure 1). Phylogenetic analyses based on combined partial TEF+RPB1+RPB2+*Bloc* sequences consisting of 59 fungal taxa resolved most *Beauveria* lineages in separate terminal branches (Figure 1). This

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1http://mafft.cbrc.jp/alignment/server/
Conidial Viability of the Beauveria bassiana Complex and Beauveria scarabaeidicola Complex Isolates

Percentage of conidial germination was used to determine conidial viability. The conidial viability of the B. bassiana complex isolates was high, but the highest value (x = 95%) was found on isolates of YFCC 844 from soil (see Table 3). The values of the conidial viability of the isolates in the B. scarabaeidicola complex were significantly lower than those in the B. bassiana complex. Only four B. scarabaeidicola complex isolates with conidial viability were greater than 65%, and their conidial viability values were not significantly different (P < 0.05).

Virulence of the Beauveria bassiana Complex and Beauveria scarabaeidicola Complex Isolates

Ten isolates had conidial viability greater than 65%. These isolates were then selected for pathogenicity tests against B. mori and T. molitor larvae as well as P. brevitarsis adults (Table 4). The B. bassiana complex isolates were shown to have great potential for use in the management of various insect pests; by contrast, the B. scarabaeidicola complex isolates showed obvious host specificity and low virulence. All tested isolates in the B. bassiana complex inflicted mycoses on B. mori and T. molitor larvae and caused over 80% mortality, whereas those in the B. scarabaeidicola complex did not. It was determined that the 10 isolates were pathogenic to P. brevitarsis adults but demonstrated different levels of virulence. Like the conidial viability, the mortalities of P. brevitarsis adults caused by the B. scarabaeidicola complex isolates were significantly lower than those of isolates in the B. bassiana complex (P < 0.05), strengthening the hypothesis that the virulence of certain entomopathogenic fungi is related to their conidial viability (Butt et al., 1994; Fernandes et al., 2007). Additionally, B. bassiana YFCC 844, which was isolated from soil and exhibited the highest conidial viability, showed high virulence against B. mori and T. molitor larvae, as well as P. brevitarsis adults, causing (94.00 ± 1.15)% mortality against B. mori larva, (95.33 ± 1.45)% mortality against T. molitor larva, and (79.00 ± 1.53)% mortality against P. brevitarsis adults (Table 4). Mycelium samples from cadavers were aseptically removed and cultured on PDA. Microscopic examination recovered the same morphological characters of conidiophores and conidia as the inoculated fungal strain. Further, TEF sequences from DNA extracted from recultures of the external mycelium of cadavers were found to match that of inoculated strain perfectly.

TAXONOMY

Beauveria polyrhachicola H. Yu & Y. Wang, sp. nov. Figure 2
MycoBank number 841450.
Etymology: “polyrhachicola” refers to the host (Polyrhachis sp.).
Sexual morph: Undetermined.

| Group                       | Taxa                                      | Marker              |
|-----------------------------|-------------------------------------------|---------------------|
| The B. bassiana complex     | B. bassiana—B. peruviensis                | 0.015               |
|                             | B. bassiana—B. polyrhachicola            | 0.019               |
|                             | B. bassiana—B. rudraprayagi              | 0.042               |
|                             | B. bassiana—B. staphylinidicola          | 0.010               |
|                             | B. peruviensis—B. polyrhachicola         | 0.011               |
|                             | B. peruviensis—B. rudraprayagi           | 0.044               |
|                             | B. peruviensis—B. staphylinidicola       | 0.015               |
|                             | B. polyrhachicola—B. rudraprayagi        | 0.045               |
|                             | B. polyrhachicola—B. staphylinidicola    | 0.019               |
|                             | B. rudraprayagi—B. staphylinidicola      | 0.045               |
| The B. scarabaeidicola      | B. scarabaeidicola—B. songmingensis       | 0.013               |
| complex                     | B. scarabaeidicola—B. subscarabaeidicola | 0.017               |
|                             | B. scarabaeidicola—B. yunnanensis        | 0.014               |
|                             | B. songmingensis—B. subscarabaeidicola    | 0.012               |
|                             | B. songmingensis—B. yunnanensis          | 0.013               |
|                             | B. subscarabaeidicola—B. yunnanensis     | 0.013               |

Morphological Features
The morphological characteristics of the three new species as well as photomicrographs of morphological structures are shown in Figures 2–4. The detailed fungal morphological descriptions are provided in the Taxonomy section.
Diameter after 14 days at 25°C smooth-walled, 1.2–2.3 µ. Odor indistinct. Vegetative hyphae septate, branched, hyaline, closely appressed to the agar surface; reverse yellowish white.

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**TABLE 3** | Conidial viability of Beauveria spp. isolates used in this study.

| Group | Species and isolate | Host/substrate | Location | Conidial viability ± SE (%)ab |
|-------|---------------------|----------------|----------|-----------------------------|
| The B. bassiana complex | B. bassiana | Lepidopteran larva | Yunnan province, China | 86.33 ± 3.48abc |
| | YFCC 841 | | | |
| | YFCC 842 | Hymenoptera: Vespidae | Yunnan province, China | 50.86 ± 0.41 |
| | YFCC 843 | Lepidoptera: Geometridae | Yunnan province, China | 36.80 ± 4.72 |
| | YFCC 844 | Soil | Yunnan Province, China | 95.00 ± 1.53ab |
| | YFCC 3369 | Coleoptera: Scarabaeidae | Yunnan province, China | 84.67 ± 3.76abc |
| | B. polyrhachicola | Hymenoptera: Formicidae | Chiang Rai Province, Thailand | 89.33 ± 0.67ab |
| | YFCC 859 | Lepidopteran larva | Chiang Rai province, Thailand | 91.67 ± 1.20ab |
| | B. rudraprayagi | | | 0 |
| | YFCC 858 | Lepidopteran larva | Chiang Rai Province, Thailand | 65.67 ± 1.20ab |
| | B. staphylinidicola | | | 0 |
| | YFCC 845 | Coleoptera: Staphylinidae | Yunnan province, China | 25.31 ± 0.69 |
| | YFCC 855 | Coleoptera: Cerambycidae | Yunnan province, China | 81.33 ± 2.33ab |

| The B. scarabaeidicola complex | B. scarabaeidicola | Coleoptera: Scarabaeidae | Yunnan province, China | 0 |
| | YFCC 846 | | | |
| | YFCC 847 | Coleoptera: Scarabaeidae | Chiang Rai Province, Thailand | 65.67 ± 1.20ab |
| | YFCC 865 | Coleoptera: Scarabaeidae | Yunnan province, China | 69.00 ± 2.65ab |
| | B. songmingensis | Coleoptera: Scarabaeidae | Yunnan province, China | 0 |
| | YFCC 848 | Coleoptera: Scarabaeidae | Yunnan province, China | 42.33 ± 0.67 |
| | YFCC 860 | Coleoptera: Scarabaeidae | Yunnan province, China | 68.00 ± 3.79ab |
| | YFCC 861 | Coleoptera: Scarabaeidae | Yunnan province, China | 0 |
| | B. subscarabaeidicola | Coleoptera: Scarabaeidae | Yunnan province, China | 18.69 ± 3.25 |
| | YFCC 863 | Coleoptera: Scarabaeidae | Yunnan province, China | 14.33 ± 2.33 |
| | B. yunnanensis | Coleoptera: Scarabaeidae | Yunnan province, China | 69.00 ± 3.46ab |
| | YFCC 862 | Coleoptera: Scarabaeidae | Yunnan province, China | 46.67 ± 1.20 |
| | YFCC 3105 | Coleoptera: Scarabaeidae | Yunnan province, China | 0 |

*Only isolates with % of conidial viability ≥ 65% were significance tested. Different lowercase letters in the same column indicate significant differences at 5% level.*

Asexual morph: Colonies on PDA reached 20–38 mm in diameter after 14 days at 25°C, white, circular, velutinous, and closely appressed to the agar surface; reverse yellowish white. Odor indistinct. Vegetative hyphae septate, branched, hyaline, smooth-walled, 1.2–2.3 µm wide. Conidiogenous cells, long cylindrical to long flask shaped, solitary or occurring in dense lateral clusters, base cylindrical to ampulliform and 1.4–3.0 µm wide, sympodially branched neck tapering into a long, slender, denticulate rachis, produced laterally on aerial hyphae or from subtending cells, 11.8–40.9 × 1.4–3.0 µm. Conidia 2.0–3.8 × 1.7–2.6 µm, Q = 1.0–1.8 (Lm = 2.7 µm, Wm = 2.1 µm, Qm = 1.3), globose, subglobose, slightly ellipsoid, oblong, or cylindrical, hyaline, aseptate, walls smooth and thin.

Type: Thailand, Chiang Rai Province, Khun Tan District (19.9233°N, 100.3133°E, 396 m above sea level), on an adult worker of *Polyrhachis* sp. emerging from leaf litter on the forest floor, May 26, 2021, Yao Wang (YHH 867, 868; living culture: YFCC 867).

Distribution: Khun Tan District, Chiang Rai Province, Thailand; Simao District, Yunnan Province, China.

Other material examined: China, Yunnan Province, Puer City, Simao District (22.7113°N, 100.9579°E, 1,360 m above sea level), on an adult worker of *Polyrhachis* sp. emerging from leaf litter on the forest floor, August 26, 2021, Yao Wang (YHH 867, 868; living culture: YFCC 867).

Notes: Regarding phylogenetic relationships, *B. polyrhachicola* forms a distinct lineage in the *B. bassiana* complex, and it is closely related to *B. peruviensis*, *B. staphylinidicola*, *B. bassiana*, and *B. rudraprayagi* (*Figure 1*). Morphologically, *B. polyrhachicola* is similar to *B. bassiana*, *B. kipukae*, *B. pseudobassiana*, *B. varroae*, and *B. peruviensis* in terms of the shape and size of the conidia (Rehner et al., 2011; Bustamante et al., 2019). However, *B. polyrhachicola* can be distinguished from them by its long conidiogenous cells (11.8–40.9 × 1.4–3.0 µm).

*Beauveria songmingensis* H. Yu & Y. Wang, sp. nov. *Figure 3* MycoBank number 841451.

Etymology: named after the location Songming County where this species was collected.

Sexual morph: Undetermined.

Asexual morph: Colonies on PDA reaching 20–35 mm in diameter after 14 days at 25°C, yellowish white, pale yellow, or shades of orange to deep orange. Odor indistinct. Vegetative hyphae septate, branched, hyaline or translucent pale yellow, smooth-walled, 2.2–4.5 µm wide. Conidiogenous
**FIGURE 1** | Phylogenetic analysis of *Beauveria* species based on combined partial TEF+RPB1+RPB2+Bloc sequences. Statistical support values (≥ 0.5/50%) are shown at the nodes for Bayesian inference (BI) posterior probabilities/maximum likelihood (ML) bootstrap support. Isolates representing ex-type material are marked with “T”. Isolates in bold type are those analyzed in this study.
cells, cylindrical to long flask shaped, solitary but usually in dense clusters of five or more, base cylindrical to ampulliform and 2.7–5.6 μm wide, apex with an indeterminate 1 μm wide geniculate, denticulate rachis, produced laterally on aerial hyphae or from subtending cells, mostly 9.6–34.1 × 2.7–5.6 μm. Conidia 3.6–6.8 × 2.8–3.9 μm, Q = 1.0–2.0 μm (Lm = 5.6 μm, Wm = 3.4 μm, Qm = 1.6), subglobose, broadly ellipsoid, ellipsoid, or oblong, hyaline, aseptate, walls smooth and thin.

Type: China, Yunnan Province, Kunming City, Songming County, Dashao Village (25.3924°N, 102.5589°E, 2,700 m above sea level), on an adult of *Pseudosymmachia flavescens* (Coleoptera: Scarabaeidae), August 12, 2018, collected by Yao Wang, (holotype: YHH 860; ex-type living culture: YFCC 860).

Distribution: at present known only in Dashao Village, Songming County, Yunnan Province, China.

Other material examined: China, Yunnan Province, Kunming City, Songming County, Dashao Village (25.3924°N, 102.5589°E, 2,700 m above sea level), on an adult of *Pseudosymmachia sp.* emerging from leaf litter on the forest floor, August 12, 2018, Yao Wang (YHH 848, 861; living culture: YFCC 848, 861).
Notes: Morphologically, *B. songmingensis* resembles the phylogenetically sister species *B. scarabaeidicola* and *B. subscarabaeidicola*. They were found to be parasitic on adult beetles (Coleoptera: Scarabaeidae), and they could be easily recognized by their distinctly yellow colony pigmentation and ellipsoid or oblong conidia. However, our morphological observation revealed a significant difference of conidia sizes between *B. songmingensis* (3.6–6.8 × 2.8–3.9 μm) and *B. scarabaeidicola* (2.5–3.5 × 1.5–2.5 μm). *B. songmingensis* differs from *B. subscarabaeidicola* by its long conidiogenous cells (9.6–34.1 × 2.7–5.6 μm) and large conidia (3.6–6.8 × 2.8–3.9 μm). Both morphological study and phylogenetic analyses of combined TEF, RPB1, RPB2, and Bloc sequence data support that this fungus is a distinctive species in the genus *Beauveria*.

*Beauveria subscarabaeidicola* H. Yu, Y. Wang & Q. Fan, sp. nov. Figure 4
MycoBank number 841452.
Etymology: “subscarabaeidicola” refers to morphologically resembling *Beauveria scarabaeidicola* but phylogenetically distinct.
Sexual morph: Stromata solitary, fleshy, pale yellow to orange, arising on adult scarab beetles buried in soil or decayed leaves, 30–45 mm long. Stipes cylindrical to clavate, yellowish white to deep yellow, 1.1–2.0 mm wide. Fertile parts clavate, being slightly wider than and indistinct from the stipes, deep yellow to orange, 5.2–26.0 mm long, 1.4–3.3 mm wide. Perithecia semi-immersed and crowded at the apex of the stromata, ampulliform, pyriform, ovoid to oblong, 265–700 × 180–320 µm (n = 50). Asci hyaline, cylindrical, 124.5–257.4 × 3.7–5.2 µm (n = 50). Apical caps prominent, hemiglobose, 2.7–3.9 µm wide, 2.4–3.2 µm high (n = 50). Ascospores hyaline, filiform, multi-septate, finally breaking into secondary ascospores, 75.6–188.5 × 1.0–1.5 µm (n = 30). Secondary ascospores cylindrical, hyaline, 6.9–11.2 × 1.0–1.5 µm (n = 50).

Asexual morph: Colonies on PDA reaching 28–42 mm in diameter after 14 days at 25°C, yellowish white, pale yellow, or light yellow, circular; reverse pale yellow, light yellow, or shades of orange to deep orange. Odor indistinct. Vegetative hyphae septate, branched, hyaline or translucent pale yellow, smooth-walled, 1.2–2.5 µm wide. Conidiogenous cells, phialidic, solitary but usually in dense clusters of five or more, base subspherical to ampulliform and 2.8–5.0 µm wide, apex with an indeterminate...
1 μm wide geniculate, denticulate rachis, produced laterally on aerial hyphae or from subtending cells mostly 4.8–6.9 × 2.0–4.6 μm. Conidia 2.6–4.2 × 1.9–3.5 μm, Q = 1.0–1.4 μm (Lm = 3.4 μm, Wm = 2.8 μm, Qm = 1.2), subglobose or broadly ellipsoid, hyaline, aseptate, walls smooth and thin.

Type: China, Yunnan Province, Kunming City, Songming County, Dashao Village (25.2398°N, 102.5617°E, 2,697 m above sea level), on an adult of Anomala exoleta (Coleoptera: Scarabaeidae), July 23rd, 2019, collected by Dexiang Tang, (holotype: YHH 863; ex-type living culture: YFCC 863).

Distribution: at present known only from Dashao Village, Songming County, Yunnan Province, China.

Other material examined: China, Yunnan Province, Kunming City, Songming County, Dashao Village (25.2398°N, 102.5617°E, 2,697 m above sea level), on an adult of Anomala exoleta, July 23, 2019, Dexiang Tang (YHH 864; living culture: YFCC 864).

Notes: Beauveria subsarabaeidicola is practically indistinguishable in morphology to B. sarabaeidicola. Our morphological observation revealed no significant differences in the morphological characteristics of teleomorph and anamorph between the two species (Kobayasi and Shimizu, 1976; Rehner et al., 2011; Shrestha et al., 2014). The lack of diagnostic morphological features to distinguish B. subsarabaeidicola and B. sarabaeidicola was overcome by delimiting the two species using DNA-based methodologies.

**DISCUSSION**

It is generally agreed that distinguishing individual Beauveria species can be difficult using only morphological characters, as several species in the genus are morphologically cryptic species. In this study, we conducted a comprehensive investigation of the cryptic species diversity of the B. bassiana complex and B. sarabaeidicola complex. The molecular phylogeny clearly suggested the existence of distinct species in the B. bassiana complex and B. sarabaeidicola complex that we accordingly propose as new species: B. polyrhachicola (Figure 2), B. songmingensis (Figure 3), and B. subsarabaeidicola (Figure 4). Beauveria polyrhachicola is practically indistinguishable in morphology from other members of the B. bassiana complex. The shape and size of the conidia and the colony color of B. polyrhachicola, among other morphological features, have been observed in B. bassiana, B. rudraprayagi, B. staphylinidicola, and B. peruviensis (Rehner et al., 2011; Agrawal et al., 2014; Kepler et al., 2017; Bustamante et al., 2019). In the B. sarabaeidicola complex, the macromorphology of B. sarabaeidicola, B. songmingensis, and B. subsarabaeidicola is very similar, and thus species cannot be distinguished visually. The macroscopic and microscopic observations performed during our investigation revealed the extensive overlap in morphological characters and the lack of distinctive phenotypic variation, supporting the notion of cryptic species in a species complex.

At present, multi-locus phylogenetic analyses have gained importance in delimiting the species within the entomopathogenic fungi Beauveria. Rehner et al. (2011) divided B. bassiana s. lat. and B. brongniartii s. lat. into several cryptic species and described six new species based on the Bloc nuclear intergenic region and three nuclear genes encoding elongation factor 1-a (TEF), RNA polymerase II largest subunit (RPB1), and RNA polymerase II second largest subunit (RPB2). Subsequently, more than seven new species and new

| Group                              | Species and isolate | Mortality ± SE (%) |
|------------------------------------|--------------------|--------------------|
|                                    |                    | B. mori larva      | T. molitor larva | P. brevitarsis adult |
| The B. bassiana complex            | B. bassiana        | 89.33 ± 0.88a     | 85.00 ± 1.73c   | 67.67 ± 1.76bc       |
|                                    | YFCC 841           | 94.00 ± 1.15a     | 95.33 ± 1.45a   | 79.00 ± 1.53a        |
|                                    | YFCC 844           | 81.33 ± 2.03b     | 91.33 ± 2.03ab  | 74.67 ± 2.60ab       |
|                                    | B. polyhachicola   | 82.00 ± 1.59b     | 84.00 ± 3.06f   | 67.00 ± 3.79f        |
|                                    | YFCC 859           | 91.33 ± 2.03a     | 87.00 ± 1.15c   | 78.67 ± 2.33h        |
|                                    | B. staphylinidicola| 80.67 ± 1.86b     | 81.67 ± 1.45c   | 62.33 ± 2.03c        |
| The B. sarabaeidicola complex      | B. sarabaeidicola  | 0                   | 0                 | 31.33 ± 3.18b        |
|                                    | YFCC 847           | 0                   | 0                 | 48.00 ± 5.20h        |
|                                    | YFCC 865           | 0                   | 0                 | 49.93 ± 4.70f        |
|                                    | B. songmingensis   | 0                   | 0                 | 49.67 ± 5.90f        |
|                                    | YFCC 860           | 0                   | 0                 | 49.33 ± 4.70f        |
|                                    | B. yunnanensis     | 0                   | 0                 | 49.67 ± 5.90f        |
|                                    | YFCC 862           | 0                   | 0                 | 49.33 ± 4.70f        |

*Corrected mortality. Different lowercase letters in the same column indicate significant differences at 5% level.
combinations were confirmed using combined analysis of the four-locus sequence data (Zhang et al., 2012; Chen et al., 2013, 2017, 2018; Agrawal et al., 2014; Robène-Soustrade et al., 2015; Imoulan et al., 2016). In more recent studies, six species were added to the genus based on multilocus (nrSSU, nrLSU, TEF, RPB1, and RPB2) sequence data: B. acidrophila, B. blattidicola, B. diapheromeripilna, B. locustipila, B. scarabaeidicola, and B. staphylinidicola (Kepler et al., 2017). In this study, we analyzed most species of the newly circumscribed genus Beauveria based on phylogenetic inferences of six nuclear molecular markers (nrSSU, nrLSU, TEF, RPB1, RPB2, and Bloc). Phylogenetic analyses based on the five-locus (nrSSU, nrLSU, TEF, RPB1, and RPB2) dataset and the combined four-locus (TEF+RPB1+RPB2+Bloc) sequences produced trees with similar topologies that resolved most Beauveria lineages in separate terminal branches (Figure 1 and Supplementary Figure 1). The results of the present work indicate that the first dataset was conducive to determining the phylogenomic relationships between Beauveria and its related genera, and the use of the latter was essential to establish robust Beauveria species boundaries, particularly the B. bassiana complex and B. scarabaeidicola complex.

Scarab beetles are leaf and root feeding pests of grasses, grains, sugarcane, strawberry, potato tubers, and young nursery plants (Crocker et al., 1996; Yokoyama et al., 1998). Based on the published literature, there are about six Beauveria spp. that parasitize adult scarab beetles: B. asiatica, B. bassiana, B. brongniartii, B. majiangensis, B. pseudobassiana, and B. scarabaeidicola (Rehner et al., 2011; Kepler et al., 2017; Chen et al., 2018; Khonsanit et al., 2020; Wang Y. et al., 2020). Here, we identified an extension of the members to also include B. malawiensis, B. songmingensis, B. subscarabaeidicola, and B. yunnanensis, as shown in Figure 1. Chen et al. (2019) emphasized that hosts of B. yunnanensis isolates were Lepidoptera pupae. However, our morphological observations of specimens from a type locality of B. yunnanensis indicated that their hosts were adult scarab beetles. Moreover, the host of B. yunnanensis was not shown in their publication (Chen et al., 2019). It seems that the host of Lepidoptera pupa is doubtful. There is reason to believe that members of the B. scarabaeidicola species complex are host-specific.

Not all scarab-killing pathogens are suitable for mycoinsecticide formulations that control scarab beetles. Our data suggested that the B. scarabaeidicola complex isolates showed low virulence. In addition, mortalities in P. brevitarsis adults caused by the B. scarabaeidicola complex isolates were significantly lower than those of isolates in the B. bassiana complex. Additional research is needed to determine the effectiveness of other species before future consideration of isolates for biological pest control.

CONCLUSION

The B. bassiana complex and B. scarabaeidicola complex, as special groups in the genus Beauveria, are rich in species diversity and have a wide distribution in nature. The B. bassiana complex, which is made up of five species, is a cosmopolitan group of soilborne necrotrophic arthropod-pathogenic fungi that have been shown to have great potential for the management of various insect pests. The B. scarabaeidicola complex is composed of pathogens specific to scarab beetles, and it is found on leaf litter or buried in soil. Species in this complex are morphologically highly similar and can hardly be distinguished macroscopically. In this study, we reported the discovery and description of three new species: B. polyrhachicola, which was found in the B. bassiana complex, and B. songmingensis and B. subscarabaeidicola, which were found in the B. scarabaeidicola complex. In addition, 10 species of Beauveria were found to be parasitic on scarab beetles. However, not all members are suitable for mycoinsecticide formulations for controlling scarab beetles. Our data suggested that the B. scarabaeidicola complex isolates showed obvious low virulence. Additionally, the mortality of Protaetia brevitarsis adults caused by the B. scarabaeidicola complex isolates was significantly lower than that of isolates in the B. bassiana complex.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in GenBank. The accession numbers can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

YW and HY: conceptualization. YW: methodology, writing—original draft preparation, and formal analysis. YW and QF: software. QF, W-QZ, and DW: validation. YW, DW, D-XT, PH, and HY: investigation. YW, D-XT, and PH: resources. HY: writing—review and editing and funding acquisition. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.841604/full#supplementary-material
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