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Beauveria lii sp. nov. isolated from *Henosepilachna vigintioctopunctata*

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Abstract — This paper describes a previously unreported entomopathogenic *Beauveria* species isolated from a lady beetle larva collected in Xunyi County, Shaanxi Province, China, in September 2010. Its main distinguishing morphological feature is its ellipsoidal to cylindrical conidia that are larger than those of other *Beauveria* species with cylindrical conidia. Phylogenetic analysis of four nuclear loci uniquely differentiate it from known *Beauveria* species that also produce cylindrical conidia, but place it with *B. varroae* and *B. kipukae*, two cryptic species that constitute the sister lineage to *B. bassiana* sensu stricto, additionally supporting its proposed species status. Integration of morphological and molecular evidence support classification of this unknown organism as a new species, here named *Beauveria lii.*

Key words — entomogenous fungi, taxonomy, multilocus phylogeny

Introduction

Beauveria Vuill. is one of the most ubiquitous anamorphic genera of entomopathogenic fungi. The genus is characterized morphologically by sympodial conidiogenous cells and sporulation on an indeterminate, denticulate rachis. Because the broad overlap in conidial dimensions and shape made it difficult to differentiate species, MacLeod's (1954) broad-sense species, *B. bassiana* (Bals.-Criv.) Vuill. and *B. tenella* (Sacc.) Siemaszko (= *B. brongniartii* (Sacc.) Petch), were used for more than 50 years until molecular evidence demonstrated that these were complex species consisting of morphologically similar but cryptic lineages (Rehner & Buckley 2005, Rehner et al. 2011). Evidence for cryptic diversification was first inferred from the nuclear ribosomal internal transcribed spacer (ITS) and elongation factor 1-alpha (EF1-α) (Rehner & Buckley 2005). Rehner et al. (2011) proposed a multilocus phylogeny of *Beauveria* species based on partial sequences of RNA polymerase II largest subunit (RPB1), RNA polymerase II second largest subunit (RPB2), translation elongation factor-1 alpha (TEF), and the Bloc nuclear intergenic

region. They established a new classification system that distinguished 12 species, six of which produce ellipsoidal or cylindrical conidia (i.e., *B. amorpha* (Höhn.) Samson & H.C. Evans, *B. malawiensis* S.A. Rehner & Aquino de Muro, *B. caledonica* Bissett & Widden, *B. brongniartii*, *B. asiatica* S.A. Rehner & Humber, *B. sungii* S.A. Rehner & Humber; Samson & Evans 1982, Bissett & Widden 1988, Rehner et al. 2006, Shimazu et al. 1988, Rehner et al. 2011). Five other species producing globose to subglobose conidia are *B. bassiana*, *B. australis* S.A. Rehner & Humber, *B. kipukae* S.A. Rehner & Humber, *B. pseudobassiana* S.A. Rehner & Humber, and *B. varroae* S.A. Rehner & Humber (Rehner et al. 2011). In addition, *B. vermiconia* de Hoog & V. Rao (de Hoog & Rao 1975) produces comma-shaped conidia. The species position of *B. sobolifera* Zuo Y. Liu et al. (Liu et al. 2001), anamorph of *Cordyceps sobolifera* (Hill ex Watson) Berk. & Broome, has yet to be verified by the multilocus method.

A fungal epizootic was observed in a population of the lady beetle *Henosepilachna vigintioctopunctata* in Shaanxi, China, caused by a *Beauveria* species. Although this organism formed colonies that were similar to those of *B. bassiana*, it had a unique microscopic morphology that differs from *B. bassiana* and other species. In this study, we investigate the morphological characteristics and molecular evidence to determine whether this organism is a novel species.

Materials & methods

Fungal culture

20 *Beauveria* strains were isolated from specimens of the lady beetle species *Henosepilachna vigintioctopunctata* (*Coleoptera*, *Coccinellidae*), collected in Shaanxi, China. RCEF5500 was selected as a test subject from these isolates. Routine growth was tested on full strength Sabouraud's dextrose agar. This isolate was incubated at 25°C under 12:12 hour light-dark fluorescent illumination.

Colony growth and morphology

Colony description and measurement were determined on the 10th day of culturing. Terms and notations used to describe colony coloration followed those of Kornerup & Wanscher (1961). Microscopic measurements of conidiogenous cells and conidia were taken from slide cultures on the 4–6th day. Images were acquired with a DP70 digital camera mounted on an Olympus BX51 microscope, with DP-BSW software (Olympus, Tokyo, Japan). Mean values for length, width, and length to width ratio (Q) are indicated by L^m, W^m, and Q^m.

DNA extraction, PCR, cloning, sequencing, and analysis

Conidia were inoculated on potato dextrose agar (PDA) Petri plates-overlaid with a disc of sterilized cellophane, and incubated at 25°C for 5 days. Fungal mycelia were obtained by scraping the cellophane. DNA extraction was performed using benzyl chloride to disintegrate the cell wall chemically as in previously described methods (Zhu et al. 1994, Wang et al. 2005). The concentration and quality of the purified DNA was evaluated by 0.8% agarose gel electrophoresis and spectrophotometry. The extracted

DNA was added to sterile distilled water at a final concentration of 100 ng per μL and stored at –20°C for the following PCR reactions.

Nuclear ribosomal internal transcribed spacer region (ITS) was amplified by PCR as described by White et al. (1990). For phylogenetic analysis of the isolated *Beauveria* strains (Rehner et al. 2011), the partial sequences of four nuclear loci (TEF, Bloc, RPB1, and RPB2) were amplified as described by Rehner & Buckley (2005), Rehner et al. (2011). PCR products were purified with the AxyPrep DNA gel purification kit (Axygene Biotechnology Hangzhou, Hangzhou, China) and were cloned using the PMD18-T plasmid vector (TaKaRa Dalian Corporation, Dalian, China).

The DNA samples were sequenced by Invitrogen Company (Shanghai, China). The derived ITS, TEF, Bloc, RBP1, and RBP2 sequences of RCEF5500 were submitted to GenBank.

Sequence alignment and phylogenetic analyses

TEF, Bloc, RBP1, and RBP2 sequences from 68 taxa determined by Rehner et al. (2011) (including 67 *Beauveria* isolates and one *Isaria tenuipes* Peck strain, which was used as the outgroup) were downloaded from GenBank. Multiple sequence alignments of these loci for the 68 isolates and from the isolate RCEF5500 was carried out by the software Bioedit (Hall 1999). Editing of sequences was performed with Bioedit, according to Rehner et al. (2011). The sequence files of the above four genes were concatenated manually into a single file and submitted to European Molecular Biology Laboratory-European Bioinformatics Institute for multiple alignment using fast Fourier transform (http://www.ebi.ac.uk/Tools/msa/mafft/). The resulting output was in Nexus file format to permit phylogenetic analysis by Molecular Evolutionary Genetics Analysis 4.0 (Tamura et al. 2007). The dataset was analyzed using both maximum parsimony and Bayesian approaches.

The combined data set of the four genes was analyzed phylogenetically using PAUP* 4.0b10 (Swofford 2003) under maximum parsimony, employing a heuristic search with 100 random addition replicates, tree bisection-reconnection (TBR) branch swapping, and saving multiple trees. Branch support was estimated by performing 1000 bootstrap replicates, with a full heuristic search (Felsenstein 1985). Clades with bootstrap values ≥70% were considered robustly supported by the data and are listed above branches in the *Beauveria* phylogenetic tree (Fig. 1).

A Bayesian analysis was carried out in MrBayes 3.1.2 (Huelsenbeck et al. 2001) applying a general time reversible model and gamma-distributed rate variation to account for rates of variation across sites, with a proportion of them being invariant sites. The remaining parameters were default values. One tree was saved to a file every 1000 generations for a total of 1,000,000 Markov Chain Monte Carlo (MCMC) generations; the first 25% of trees were discarded as burn-in. Values of the posterior probabilities for the branches supported by ≥95% of pseudo-replicates were listed below branches in the phylogenetic tree.

Results

The derived ITS, TEF, Bloc, RBP1, and RBP2 sequences of RCEF5500 were accepted by GenBank with accession numbers of JN689372, JN689371, JN689373, JN689374, and JN689370, respectively.

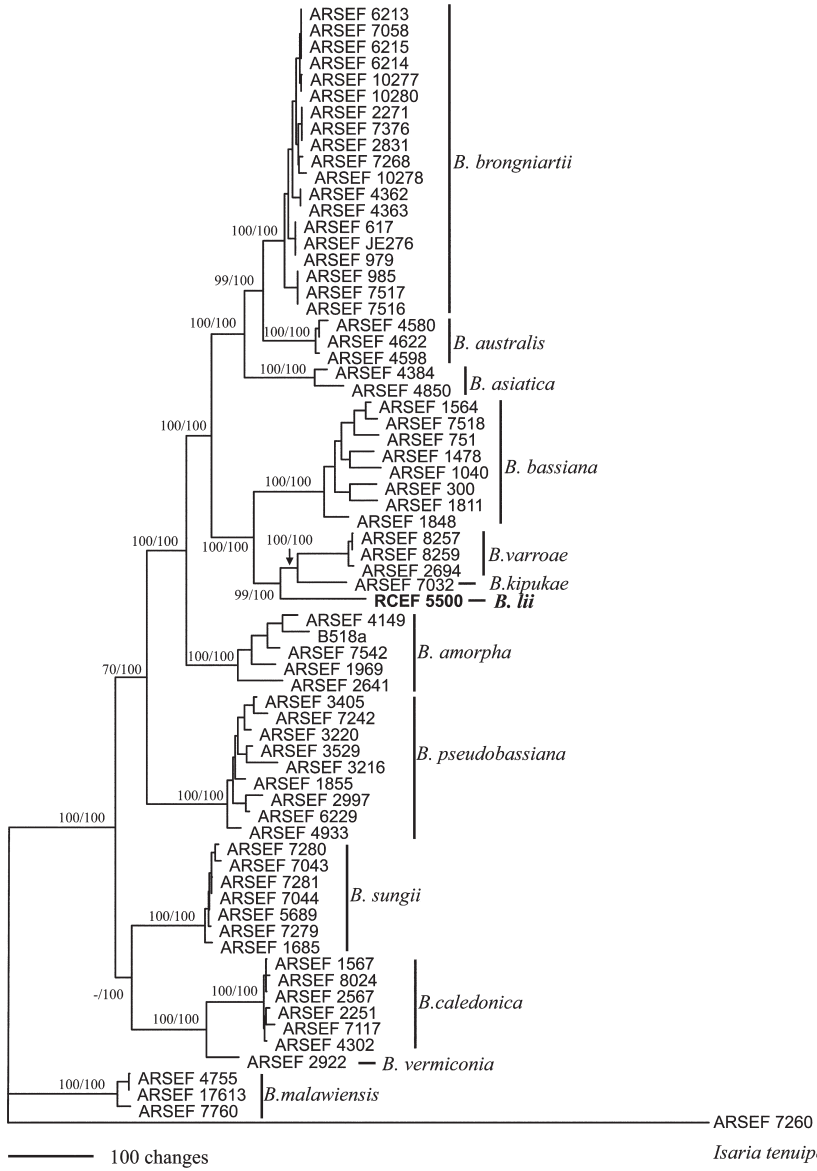

Fig. 1. Phylogenetic tree of *Beauveria* based on maximum parsimony and Bayesian analysis of the combined Bloc, TEF, RPB1, and RPB2 dataset. Bootstrap values (≥70%) and Bayesian posterior probabilities (≥95%) are labeled above and below branches, respectively. Sequence data from all strains except RCEF5500 are from Rehner et al. (2011). RCEF5500 is indicated in boldface.

The ITS sequence of RCEF5500 differed from other *Beauveria* species. Online BLAST searches of the National Center for Biotechnology Information databases showed that the RCEF5500 isolates are most similar to *B. varroae* (ARSEF 8257, HQ880800, 98%), *B. kipukae* (ARSEF 7032, HQ880734, 97%), and *B. bassiana* (ARSEF 1564, HQ880761, 97%), making it hard to determine the species position. Further comparative multilocus analysis was undertaken to determine its taxonomic and phylogenetic status.

The combined four-gene, 69 taxon data set published by Rehner et al. (2011) included 7750 bp of sequence data (Bloc: 1645; TEF: 1010; RPB1: 2928; RPB2: 2167). After excluding ambiguously aligned regions, the final alignment comprised 7569 bp (Bloc: 1558; TEF: 986; RPB1: 2861; RPB2: 2164), of which 1261 were parsimony informative. The sequence alignments are available from TreeBASE as submission 11144. The bootstrap values from maximum parsimony analyses and the posterior probability values from one of the Bayesian analyses are shown in Fig.1.

Phylogenetic analysis with four nuclear loci supports RCEF5500 as closely related to two species, *B. varroae* and *B. kipukae* but in an independent lineage (Fig. 1). Morphologically, RCEF5500 is easily distinguishable from these two species by its large, ellipsoidal to cylindrical conidia. Additionally, the phylogenetic analysis showed that RCEF5500 and three morphologically similar species (*B. amorpha, B. caledonica, B. malawiensis*) all clustered separately into different clades and are clearly distinguishable from each other. Therefore, the phylogenetic data support the recognition of RCEF5500 as a distinct species.

Taxonomy

Beauveria lii Sheng L. Zhang & B. Huang, **sp. nov.** Plates 2–11

MycoBank MB563695

Differs from all other *Beauveria* species by its larger ellispoidal to cylindrical conidia, with a larger mean length/width ratio.

Type: China, Shaanxi Province, Xunyi County, isolated from a larva of *Henosepilachna vigintioctopunctata*, 23 Sep 2010, coll. Ling-ming He (**Holotype**, RCEF5500; GenBank JN689370–689374). A living ex-type culture ARSEF 11741 is deposited in USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY).

Etymology: *lii* is named in honor of Zengzhi Li, in recognition of his contributions to the study of entomopathogenic fungi, especially *B. bassiana*.

Colony growth and appearance similar on full strength Sabouraud's dextrose and potato dextrose agars, 28–29 mm in diam. after 10 d at 25°C, non-odorous, cottony, powdery while sporulating; white to cream, with a white margin. Reverse colorless, sometimes pale yellow in older portions. On Czapek-Dox agar, colonies slow growing, 19 mm in diameter, thin, sparse, with reverse colorless; sporulating poorly. Vegetative hyphae septate, branched, hyaline, smooth walled, 1.3–3.4 μm wide. Conidiogenous cells solitary but occasionally

occurring in tight clusters of two to three, the base ellipsoidal to cylindrical 4.8–9.0 × 1.7–2.6 µm (Figs 2, 4, 7), or sometimes globose to subglobose (Figs 5, 6) and produced laterally on aerial hyphae or from subtending cells mostly 4.8–9.0 × 1.7–2.6 µm (Figs 3, 10) Conidiogenous cell apex with an indeterminate, geniculate, denticulate rachis less than 1µm wide. Conidia (3.1–)4.3–6.5(–10.1) × (1.4–)2.1–2.6(–3.6) µm, Q = 2.1–2.7 (L^m = 5.2 µm, W^m = 2.3 µm, Q^m = 2.3), mostly ellipsoidal to cylindrical (Fig. 8), occasionally obovoid (Figs 6, 10) and very occasionally slightly flattened on one side, usually produced from conidiogenous cells but occasionally directly from hyphal tips or laterally from hyphae (Figs 9, 11). Teleomorph not observed.

Additional material examined: RCEF5183–5195, RCEF5887–5862.

Microscopically, *B. lii* is distinct from other *Beauveria* species mainly by its large ellipsoidal to cylindrical conidia. The six other *Beauveria* spp. with similarly shaped conidia all produce smaller conidia: *B. amorpha* (mean = 3.3 × 2.0 µm), *B. asiatica* (mean =3.2 × 2.3 µm), *B. brongniartii* (mean = 3.0 × 1.6 µm), *B. caledonica* (mean = 3.6 × 1.8 µm), *B. malawiensis* (mean = 3.9 × 1.9 µm), *B. sungii* (mean = 3.0 × 2.1 µm); the mean conidial length/width ratios (Q^m) of these six species are also smaller (ranging from 1.4 to 2.1; Rehner et al. 2011, Humber & Rehner 2007) than that of *B. lii,* which has the largest Q^m in the genus.

Discussion

In addition to the cryptic species within the *B. bassiana* sensu lato and *B. brongniartii* sensu lato, additional new species (e.g., *B. asiatica*, *B. australis*, *B. kipukae*, *B. pseudobassiana*, *B. sungii, B. varroae*) have been differentiated chiefly on the strength of molecular data. However, morphological identification of these species remains difficult in the absence of molecular data. For example, morphological overlap makes difficult the physical differentiation among *B. bassiana, B. pseudobassiana*, *B. australis*, *B. kipukae*, and *B. varroae*. The multilocus phylogeny approach should be applied to identification of both cryptic and unknown species of *Beauveria*. This study has demonstrated that the four-locus phylogenetic method of Rehner et al. (2011) can be used to provide a practical solution for distinguishing complex species and establishing a taxonomic and systematic position for an unknown species, such as *B. lii*.

China is a country rich in fungal species diversity; various *Beauveria* species have been recorded in different geographical locations, including the dry northwest region. *Beauveria lii* was discovered in such an area, suggesting that this vast zone should be further investigated for entomopathogenic fungi.

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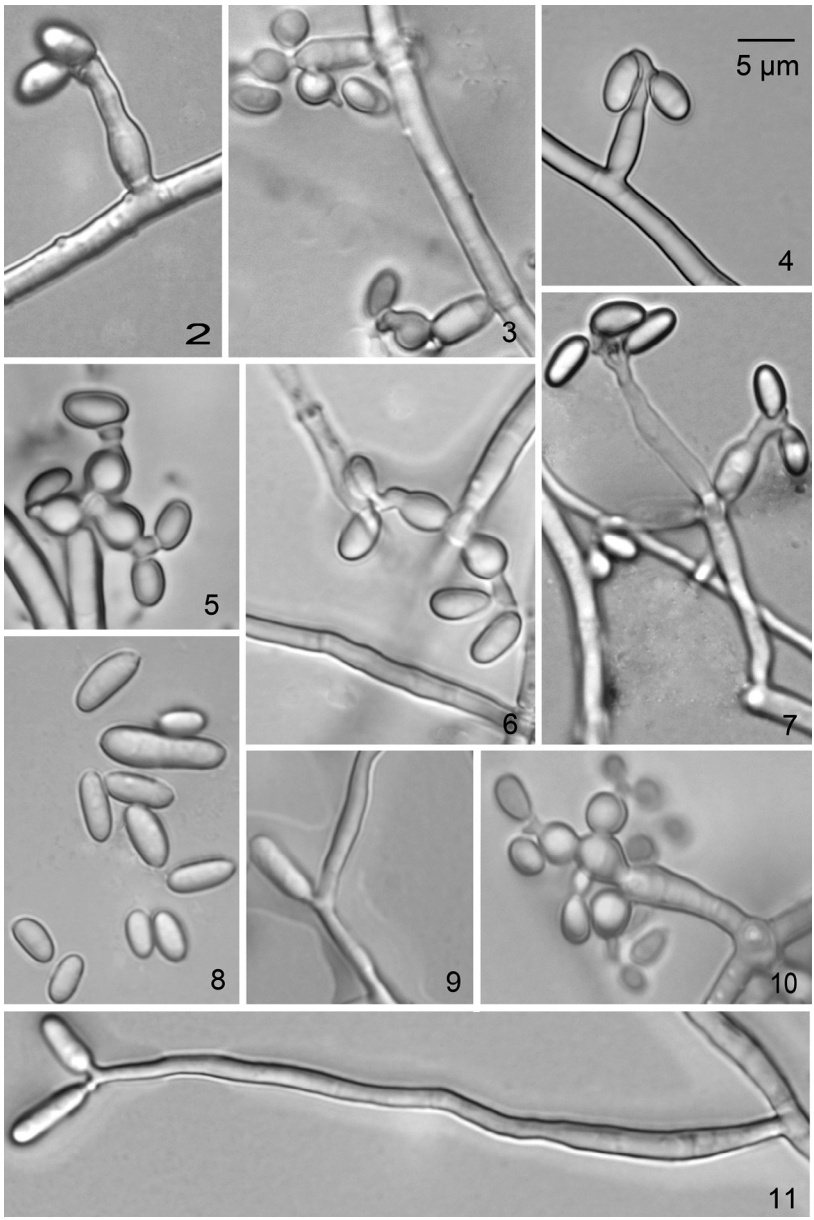

Figs 2–11. *Beauveria lii* (holotype, RCEF5500). 2, 4, 7. Ellipsoidal or cylindrical conidiogenous cells. 3, 10. Globose conidiogenous cell originating from subtending cells. 5–6. Globose conidiogenous cell. 8. Conidia. 9. Conidia originating from mycelium directly. 11. Conidia forming from a hyphal tip. Bar = 5 µm.

identification. This work was supported by a grant from the Natural Science Foundation of China (Nos. 30972368 and 31070009).

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