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**The integrative taxonomy of *Beauveria asiatica* and *B. bassiana* species complexes with whole-genome sequencing, morphometric and chemical analyses.**

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**Abstract**

Fungi are rich in complexes of sibling species that need combinations of approaches to be delimited, including with genomic information. *Beauveria* (Cordycipitaceae, Hypocreales) is a well-known genus of entomopathogenic fungi, used as a biocontrol agent. We present in this study a polyphasic taxonomy regarding two widely distributed complexes of *Beauveria*: *B. asiatica* and *B. bassiana* sensu lato. Some of the genetic groups as previously detected within both taxa were either confirmed or fused using population genomics. High levels of divergence were found between two clades in *B. asiatica* and between three clades in *B. bassiana*, supporting their subdivision as distinct species. Morphological examination focusing on the width and the length of phialides and conidia showed no difference between the clades within *B. bassiana* while the length of conidia was significantly different between the clades within *B. asiatica*. The secondary metabolites profiles obtained by liquid chromatography-mass spectrometry (LC-MS) allowed a distinction between *B. asiatica* and *B. bassiana*, but not between the clades therein. The maximum-likelihood reconstruction of ancestral host association by mapping information of the hosts' taxonomic order onto the whole-genome phylogeny established that the common

ancestor to *Beauveria* was predominantly associated with *Coleoptera* while the common ancestors to one clade of *B. bassiana* was predominantly associated with *Orthoptera*, and that of another clade in *B. asiatica* was partially associated with *Lepidoptera*. Based on these genomic, morphological, chemical and ecological data, we proposed a clade of *B. asiatica* as a new species, named *B. thailandica*, and two clades of *B. bassiana* to be respectively *B. namnaoensis* and *B. neobassiana*. Such closely related but divergent species with different host ranges have potential to elucidate the evolution of host specificity with application in biocontrol.

## Introduction

Fungi are rich in complexes of sibling species, i.e. morphologically similar but genetically isolated. In pathogenic fungi, sibling species are often specialized on different hosts (Le Gac et al. 2007; Giraud et al. 2010; Kobmoo et al. 2012), so that it is essential to elucidate their genetic subdivision and the species limits. Taxonomy is therefore an essential tool for understanding the evolution of host specificity in pathogens and to evaluate the risk of attacking untargeted species for biocontrol agents.

*Beauveria* (Cordycipitaceae, Hypocreales) is a well-known genus of entomopathogenic fungi first discovered in the 18<sup>th</sup> century (Basalmo-Crivelli 1835) with *B. bassiana* as the type species (Vuillemin 1912). It is widely distributed worldwide (Rehner & Buckley 2005; Imoulan et al. 2017). Being recognized as a rich source of efficient mycoinsecticides, in particular *B. bassiana* and *B. brongniartii* (Zimmermann 2007), *Beauveria* fungi have received a lot of attention as potential biological control agents (García-Estrada et al. 2016). The discovery of new species and their precise delimitation contributes to enlarge potential for finding suitable agents and to their safe use. The genus is characterized by the formation of short, sympodial, globose to flask-shaped phialides with holoblastic conidia (Khonsanit et al. 2020). The size and the shape of conidia are variable within the genus and historically constituted discriminant characters for delimitating and identifying species (Rehner & Buckley 2005; Imoulan et al. 2017; Abdessamad 2019). However, these morphological characters can exhibit overlapping values between species, even between relatively distant ones (Khonsanit et al. 2020). Furthermore, many new species have been proposed these recent years in *Beauveria*, mainly based on the monophyly of clades in molecular phylogenies, but

sometimes with only a few samples, without clear morphological distinction (Chen et al. 2018). *Beauveria* is thus a genus with potential cryptic species to be discovered. However, there has never been a thorough morphometric analysis with solid statistical tests.

Although *Beauveria* fungi are known to produce an array of secondary metabolites (Xu et al. 2008, 2009; Rohlf & Churchill 2011; Udompaisarn et al. 2020), chemical compounds produced have been rarely used for taxonomic purpose in this genus; the potential of using chemical diversity as markers for classification and species identification was explored a long time ago for *Beauveria* (see Mugnai et al. 1989; Bridge et al. 1990) without any recent update for recently discovered species. Chemical compounds allowed the distinction between populations within *B. bassiana* (Bridge et al. 1990) but this finding was not compared with genetic data.

The abundant genomic resources available now for *Beauveria* (Valero-Jiménez et al. 2016; Toopaang et al. 2017; Mei et al. 2020), combined with decreasing cost for whole-genome sequencing (WGS), make it possible to access novel genetic information on a large number of samples with reasonable cost and time. Next generation sequencing (NGS) has in fact been used to reveal cryptic species with success these recent years (Sepúlveda et al. 2017; Kobmoo et al. 2019; Matute & Sepúlveda 2019).

In the present study, we aimed at revising the taxonomy of two important *Beauveria* taxa, *B. bassiana* and *B. asiatica*. In the past, populations of *B. bassiana* were studied using limited numbers of markers (Wang et al. 2003, 2005; Mitina et al. 2011; Meyling et al. 2012), some of which are now considered unreliable, such as Amplified Fragment Length Polymorphism (AFLP) (Aquino De Muro et al. 2005; Trissi et al. 2013). A recent population genomics study of this species showed clear intraspecific genetic groups but their taxonomic status was not evaluated with appropriate phylogenetic frameworks or detailed examination of their morphology and chemistry (Mei et al. 2020).

In our previous taxonomic revision of the genus, we found poorly supported monophyletic clades based on multigene phylogenies within *B. asiatica* (four clades named A, B, C and D) and *B. bassiana* (three clades named A, B and C) (Khonsanit et al. 2020). The poor node supports however did not allow the recognition of these clades as distinct species and morphological traits appeared overlapping. The poor node support may have been due to the limited number of markers. In the current

study, our objectives were to evaluate the taxonomic status of the genetic groups within *B. asiatica* and *B. bassiana* by combining various data including whole-genome sequencing (WGS), as well as statistical analyses of morphological characters (the width and the length of conidia and phialides) and chemical profiles determined by liquid chromatography-mass spectrometry (LC-MS). A solid taxonomic revision should indeed be based on a combined dataset including a sufficient amount of genetic information, as well as morphological and chemical traits on a high number of individuals. This constitutes the foundation of integrative (or holistic) taxonomy of fungi.

## **Materials and Methods.**

### ***Fungal culture and DNA extraction.***

Seventy-eight strains of presumably 12 *Beauveria* species from BIOTEC culture collection (BCC) and ARS collection of entomopathogenic fungal cultures (ARSEF), including the type strains of known species and strains previously identified as *B. bassiana* or *B. asiatica* as well as some unidentified strains following Khonsanit et al. (2020), were used in this study (Table S1). The strains were cultured in 50 ml of potato dextrose broth and incubated at 25 °C for a week. Fungal mycelia were harvested by filtering with a sterilized nylon mesh and washed with ethylene diamine tetraacetic acid (EDTA) and distilled water. DNA extraction was done using a cetrimonium bromide (CTAB)-based method following Kobmoo et al. (2018); the DNAs were purified using the high pure PCR template preparation kit (Roche). The quality and quantity of DNA were verified using Nanodrop™ One Microvolume UV-Vis Spectrophotometry (Thermo Fisher) and an electrophoresis on 0.8% agarose gel at 100V for 1/2h.

### ***DNA library construction and sequencing***

For whole genome shotgun sequencing of 78 strains, approximately 300 ng of each DNA sample was used for a library construction following the protocol in the MGIEasy FS library prep kit (MGI Tech, Shenzhen, China). The samples were pooled and sequenced in a single lane. Paired-end (150 bp) sequencing was performed on the MGISEQ-2000RS (MGI Tech, Shenzhen, China) according to the manufacturer's instructions.

### **Data processing and detection of single nucleotide polymorphisms (SNPs)**

Using the MegaBOLT V1.5.6.11 software package, raw reads were demultiplexed according to their barcodes and the adapter/barcode sequences were removed. After removing the low-quality regions, clean reads were mapped to the *B. bassiana* reference genome ARSEF8028 (Valero-Jiménez et al. 2016) using the MegaBOLT V1.5.6.11 alignment software (Minimap2 version 2.11-r797-v03) and the variants were called using GATK HaplotypeCaller version 3.8. SNP markers with poor quality data were filtered out using the following criteria: (a) a minor allele frequency <0.1; (b) depth coverage less than 10x; (c) more than 20% missing data. The ploidy was set as haploid as all the strains were collected from asexual mycelia, i.e. putatively haploid for this ascomycete fungus. The raw reads data were deposited at NCBI Sequence Read Archive (SRA....) associated to the BioProject accession.... The SNPs data were deposited at.....

### **Population structures, genetic diversity and linkage disequilibrium**

The selected SNPs (729,549 SNPs) using RaxML v.8 (Stamatakis 2014), by specifying GTRCAT model with Lewis biases ascertainment correction. The reliability of the resulting tree was evaluated with 1000 bootstraps. Bayesian clustering analyses were conducted with FastStructure (Raj et al. 2014) and a principal component analysis (PCA) was achieved using the package *adegenet* in R (Jombart & Collins 2015). These analyses were intended to give an initial insight into interspecific divergence and intraspecific population structures, in order to assess the taxonomic status of the samples included as well and to reveal any genetic group that can potentially be identified as new species. We calculated the fixation index ( $F_{st}$ ) and metrics of absolute divergence ( $D_{xy}$ ) between *B. asiatica* and *B. bassiana*, as well as between intraspecific genetic clusters as revealed by previous analyses. The nucleotide diversity ( $\pi$ ) within species and genetic clusters were also calculated using the PopGenome package in R (Pfeifer et al. 2014). The  $r^2$  (square of correlation coefficient representing statistical association between pairwise SNPs) among isolates belonging to *B. asiatica*, *B. bassiana* and their respective intraspecific genetic clusters were calculated between pairs of SNPs using VCFtools (Danecek et al. 2011). The decay of linkage disequilibrium was visualized using R. Neighbor-net phylogenetic network based on p-distance was constructed using the software SplitsTree v4.14.18 (Huson & Bryant 2006).

## **Species Tree and Ancestral Host Association Reconstruction**

Gene sequences were obtained using *FasterAlternateReferenceMaker* tool from GATK (McKenna et al. 2010) which altered the reference sequences with SNPs and simple indels; all complex substitutions were masked as Ns to reduce ambiguous alignment. The sequences were then aligned using the software MAFFT (Kato & Standley 2013). A total of 1,132 genes were first selected based on 1) their size (1 Kbp minimum and 10 Kbp maximum) to ensure an absence of recombination within genes, on 2) their physical distance on the genome; only genes with at least 20 Kbp between the start and the end of consecutive genes were selected to avoid the linkage disequilibrium, on 3) the number of SNPs per bp ( $> 0.02$ ) to provide sufficient phylogenetic signal. The alignments of the 1,132 genes were subjected to Bayesian analyses with MrBayes (Ronquist et al. 2012), each with 2 Markov chain Monte Carlo (MCMC) runs for 1 million generations with a 20% burn-in phase, allowed to sample across the substitution model space. Only the final gene trees with  $< 0.02$  split frequencies were retained for 1034 genes. The 1034 genes sequences were also concatenated and subjected to MrBayes with the model GTRGAMMAI, as chosen by ModelTest-NG (Darriba et al. 2020), for 5 millions of MCMC generations. The final tree was obtained after a 25% burn-in phase. The taxonomic classification corresponding to the order of hosts was mapped onto the fungal species tree based on concatenated genes, and the ancestral host associations were reconstructed based on maximum likelihood reconstruct module in MESQUITE (Maddison & Maddison 2019).

## **Bayesian Concordance Analyses (BCA)**

To evaluate whether the genetic clusters within species were consistently recovered by different markers throughout the genome, Bayesian concordance analyses (BCA) were conducted for the three clusters of *B. bassiana* with ARSEF7032 (*B. kipukae*) as an outgroup, and for the two clusters of *B. asiatica* with ARSEF617 (*B. brongniartii*) as an outgroup. By focusing on each taxon of interest with its respective outgroup, the number of genes with variable SNPs naturally reduced. The BCA were thus done by selecting, among the 1034 concatenated genes, only those which had at least two SNPs with no missing data and the number of SNPs per bp  $> 0.001$ , resulting in 887 genes for *B. asiatica* and 100 genes for *B. bassiana*. The single-gene trees obtained from Bayesian inferences were processed into BUCKy (Larget et al.

2010) which evaluated the concordance between the selected loci for the clades by giving genome-wide concordance factors with 95% interval which can be 0 (absence of concordance) to 1 (total concordance).

### 209 **Secondary Metabolites Profiling**

210 Strains of *Beauveria*, selected to represent the various genetic groups within *B. asiatica* and *B. bassiana* as well as type strains of other species (Table S1), were grown in 200 mL of yeast with malt extract and glucose (YMG) medium (10 g of malt extract, 4 g of D-glucose, 4 g of yeast extract and 1,000 ml of distilled water, pH 6.3), incubated at 25 °C under shaking condition (140 rpm). Since growth rate varies among strains, the prolonged fermentation period for an individual strain was set to be half the time required for that particular strain to reach glucose depletion. The glucose content of each fermented broth was estimated using urine glucose test strips (DIRUI®, Jilin, China). The mycelia were then separated from the broth either by filtration or centrifugation.

220 The extraction of fungal secondary metabolites was performed according to Phainuphong et al. (2017) with culture filtrates passing through successive extraction using acetone, ethyl acetate and methanol, resulting in a final methanol-based cell extract (CE). The experiment of liquid chromatography-mass spectrometry (LC-MS) was done using ultra-high performance liquid chromatography (UHPLC) - Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific, Massachusetts, USA), equipped with electrospray ionization (ESI) source. The separation of compounds was done on an Acquity UPLC® HSS T3 C18 column (1.8 µm diameter, 2.1 x 100 mm) maintained at 40 °C with flow rate of 0.4 ml/min. A mobile phase system was solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The optimized gradient for the best separation was 0.0-12.0 min, 12%-95%B; 12.0-14.0min, 95%-95%B; 14.0-16.0 min, 12%-12%B. The mass spectrometer was operated in ESI positive which covered more than 80% of metabolites present in the tested pool sample. Each sample was injected in triplicate. The MS calibration was conducted using Pierce LTQ Velos ESI Positive Ion Calibration (PSP3A 88323) and Pierce ESI Negative Ion Calibration (PSP3A 88324) according to manufacturer's protocol.

236 The software Compound Discoverer 3.1 was used for data-preprocessing steps. 237 The MS raw files were subjected to peak alignment, peak picking, adduct grouping 238 and normalization with parameters adjusted to fit to the chromatographic data obtained



from this experiment. The area under curve (AUC) of each metabolite was determined and normalized with that of pooled samples. The features (m/z at specific retention time (rt)) with relative standard deviations over 30% and average group area in non-inoculated YMG media over  $5 \times 10^5$  were filtered to ensure good-quality peaks and to cover peaks apart from culture media. Top 200 highest abundant features in the CE pool sample were selected for statistical analyses including a Euclidean distance-based neighbor-joining (NJ) tree and principal component analysis (PCA) using respectively the packages “ape” (Paradis & Schliep 2019) and “FactoMineR” (Lê et al. 2008) in R (R Core Team 2020).

### ***Morphological examination and species description***

A thorough morphological investigation was conducted following the techniques described in Khonsanit et al. (2020). The samples and culture plates were photographed by using a digital Nikon D5100 camera. Colony characteristics and microscopic measurements of phialides and conidia (length and width) were done after growth on potato dextrose agar medium (PDA, 20 g Difco potato dextrose agar, 1 L distilled water) and incubation under white light/dark cycles at room temperature; 30 phialides and conidia were measured for each strain. To statistically test the difference between the genetic groups within each of *B. asiatica* and *B. bassiana*, five strains for each genetic group were examined for the width and the length of conidia and phialides (Table S2). The data were analyzed with one-factor ANOVA for testing difference between species, as well as between the genetic groups within each species.

## **Results**

### ***Population Structure and Molecular Diversity***

The maximum-likelihood tree inferred from all the 729,549 SNPs showed clear differentiation between *B. asiatica* and *B. bassiana* as expected, and also intraspecific genetic subdivision (Fig.1a). Two and three clades could be distinguished within *B. asiatica* and *B. bassiana* respectively. One of the two clades from *B. asiatica* corresponds to the Clade C as found in Khonsanit et al. (2020), including the type strain ARSEF 4850; the other clade comprised a mix of individuals from the Clades A,

B, C and D from that previous study, therefore named here as “Clade Mixed”. The three clades from *B. bassiana* corresponded to the Clades A, B and C established in this species by Khonsanit et al. (2020); the Clade C includes the type strain ARSEF 1564. The principal component analysis (PCA) also showed clear difference as expected between *B. asiatica* and *B. bassiana* as well as between these two species and the others included (Fig.1b). Not enough individuals had been analysed from the other species to assess their differentiation one from each other. The Bayesian clustering within each of the two species recovered the same genetic clusters as above (Fig.1c and d) but, for *B. asiatica*, an additional subdivision was observed within Clade Mixed (Fig.1c). The PCA also confirmed the strong differentiation between the clades identified in the analyses above (Fig.1e-f) as well as the subdivision within *B. asiatica* Clade Mixed (clusters hereafter called mix1 and mix2: Fig.1f). These sub-clusters included mixes of strains from various genetic groups from Khonsanit et al. (2020). The various analyses thus consistently indicated the existence of intraspecific subdivision. The only exception was the strain NHJ10436 which clustered with *B. bassiana* Clade B in the Bayesian clustering and the tree, but had intermediate coordinates on the PCA (Fig.1b and 1e). This is probably due to missing data as this strain only carried 14,427 SNPs (1.97 % from total SNPs). This strain was though kept in our analyses as it personally interested us for use in biocontrol.

The  $F_{ST}$  between the two species was high (0.875) as expected for interspecific differentiation. The nucleotide diversity ( $P_i$ ) was higher in *B. bassiana* (0.107) than *B. asiatica* (0.057); this may be due to the use of *B. bassiana* genome as reference to call SNPs. The differentiation levels between clades within species were also very high, with  $F_{ST}$  reaching more than 0.7 (Table 1), but the  $F_{ST}$  between the sub-clusters mix1 and mix2 within Clade Mixed of *B. asiatica* was relatively weak (0.262). The divergence estimated by  $D_{xy}$  was also relatively high between clades, close to or higher than 0.1 (Table.1), but that between the sub-clusters mix1 and mix2 was much lower (0.041). The nucleotide diversity ( $P_i$ ) was higher in *B. asiatica* Clade Mixed (0.024 and 0.036 for the sub-clusters mix1 and mix2 respectively) than in the Clade C (0.009). In *B. bassiana*, Clade B had the highest  $P_i$  (0.031), followed by Clade C (0.018) and Clade A (0.003).

**Table 1.** Genetic differentiation ( $F_{ST}$ : below the diagonal), genetic divergence ( $D_{xy}$ : above the diagonal) between clades of *B. asiatica* and *B. bassiana*, and nucleotide diversity ( $P_i$ : the diagonal) within clades.

		<i>B. asiatica</i>		<i>B. bassiana</i>		
		Clade C	Clade Mixed	Clade A	Clade B	Clade C
<i>B. asiatica</i>	Clade C	0.009	0.0875	0.651	0.675	0.669
	Clade Mixed	0.742	0.036	0.630	0.654	0.648
<i>B. bassiana</i>	Clade A	0.991	0.969	0.003	0.157	0.119
	Clade B	0.970	0.949	0.893	0.031	0.161
	Clade C	0.980	0.958	0.913	0.848	0.018

### Phylogenetic Network and Linkage Disequilibrium

The Neighbor-Net network inferred from p-distances between the strains showed clear separation between clades within each species, with minimal reticulation between them, confirming lack of gene flow among clades (Fig.S1). The sub-cluster mix1 was nested within the sub-cluster mix2 for *B. asiatica* Clade Mixed. Reticulations were observed within *B. asiatica* Clade C and Clade Mixed. For *B. bassiana*, reticulation events could be observed only within Clade B. In all the clades within species, the  $r^2$  dropped to rapidly reach a plateau except *B. bassiana* Clade A which sustained a longer LD with  $r^2$  dropping under half the maximum around 300 Kbp (Fig.S1). These results are consistent with the absence of reticulation in the *B. bassiana* Clade A, for which the nucleotide diversity was also the lowest (0.002: Table 1).

### Phylogenetics Species Recognition

The species tree inferred from 1034 concatenated genes gave strongly supported monophyletic clades corresponding to the genetic subdivisions within *B. asiatica* (Clade C and Clade Mixed) and *B. bassiana* (Clades A, B, C) (Fig.2). The subcluster mix 1 within *B. asiatica* Clade Mixed was recovered as a well-supported monophyletic clade but not the subcluster mix 2, further supporting the view that mix1 is a lineage having originated from mix2. To evaluate whether these genetic subdivisions could be considered as distinct species using phylogenetics species criteria, we conducted Bayesian concordance analyses between genes having no missing data for *B. asiatica* with its outgroup *B. brongniartii* (ARSEF617) (887 genes),

and for *B. bassiana* with its outgroup *B. kipukae* (ARSEF7032) (100 genes). We found that the Clades A, B, C within *B. bassiana* were highly concordant throughout the genome with high Bayesian concordance factors (BCF), NHJ10436 being well anchored in Clade B. For *B. asiatica*, Clade C had a high BCF ( $> 0.8$ ) but Clade Mixed had a low BCF (0.201) (Fig.2).

### **Morphological variation**

The average among measures per strain of the length and the width of phialides and conidia are reported in Table S2. The length of phialides and conidia were significantly different between *B. asiatica* and *B. bassiana* (phialide length:  $F = 16.858$ ,  $p\text{-value} = 4.324e^{-04}$ ; conidia length:  $F = 79.566$ ,  $p\text{-value} = 6.309e^{-09}$ ) but the other traits were not significantly different between the two species (phialide width:  $F = 0.199$ ,  $p\text{-value} = 0.659$ ; conidia width:  $F = 79.566$ ,  $p\text{-value} = 6.309e^{-09}$ ) (Fig.3a). Between Clade C and Clade Mixed of *B. asiatica*, only the length of conidia was significantly different ( $F = 7.219$ ,  $p\text{-value} = 0.02$ , Fig.3b). For *B. bassiana*, none of the examined traits was significantly different between clades (Table S3) (Fig.3b).

### **Chemotaxonomy**

Metabolite profiles obtained with LC-MS are shown in Fig.S2-S3. Principal component analyses (PCA) and the Euclidean distance-based tree showed a relatively clear separation between *B. asiatica* and *B. bassiana* with some exceptions (Fig.4); the two strains MY11641 and NHJ13734 of *B. asiatica* showed a chemical similarity to *B. bassiana*. The other species included in the chemical analyses were not all well discriminated from *B. asiatica* and *B. bassiana*. On the NJ tree, *B. caledonica*, *B. pseudobassiana*, *B. sungii* and *B. vermiconia* clustered within *B. asiatica* (Fig.4a). Based on the PCA, *B. amorpha*, *B. malawiensis*, *B. pseudobassiana*, *B. varraoe* could be well distinguished from both *B. asiatica* and *B. bassiana*. In contrast, *B. gryllotalpidicola* and *B. kipukae* appeared nested within *B. bassiana* while *B. caledonica*, *B. sungii*, *B. vermiconia* were found very close to *B. asiatica* (Fig.4b, top panel). The intraspecific clades were not well separated based on these methods.

### **Ancestral host association**

The ancestral reconstruction of host association showed that *Beauveria* is definitely a genus of generalists, with a predominance for *Coleoptera* as the insect

host order associated with its common ancestor. (Fig.5). The ancestral hosts of *B. asiatica* and *B. bassiana* were inferred to be also most likely coleopteran insects. However, *B. asiatica* Clade Mixed had a notable likelihood for *Lepidoptera* while the ancestral host association of *B. bassiana* Clade A was most likely *Orthoptera*.

### **Taxonomy**

Based on genomic, morphological and ecological results above, *B. asiatica* Clade Mixed is proposed as *B. thailandica*; *B. bassiana* Clade A and Clade B are proposed as *B. namnaoensis* and *B. neobassiana*, respectively.

***Beauveria namnaoensis*** Khons., Kobmoo & Luangsa-ard, *sp. nov.* — MycoBank MB No. 838940; Fig. 6

**Etymology.** The name derived from the place where the type specimen was found, Nam Nao National Park, Nam Nao District.

**Holotypus.** THAILAND, Phetchabun Province, Nam Nao District, Nam Nao National Park, Headquater Nature Trail, 16.74°N, 101.57°E, on adult of *Xylocopa latipes* (Hymenoptera:Apidae), 4 Jul. 2012, A. Khonsanit, K. Tasanathai, P. Srikitikulchai, S. Mongkolsamrit, W. Noisripoom (holotype BBH36158; ex-holotype strain BCC64218).

**Sexual morph** — Unknown.

**Asexual morph** — Specimens found on blue milkweed beetle, grasshopper, moth, lepidopteran pupa, stink bug and wasp. Hosts covered with white mycelium, powdery when sporulating. *Phialides* hyaline, solitary, smooth-walled, base subspherical to lageniform, ampulliform, globose, subglobose, pyriform, clavate, mucronate, (2)2–3.2(5) × (1.5)1.9–2.7(3.5) µm (*n* = 150). *Conidia* hyaline, smooth-walled, globose to subglobose, occasionally obovoid, (1.5)1.9–2.8(4) × (1.5)1.7–2.3(3) µm (*n* = 150).

*Colony characteristics* — Colonies growing at room temperature attaining a diam of 29–31 mm in 10 d, and 42–43 mm in 20 d. Surface mycelium dense, floccose, sporulation starts at 4 d after inoculation, white with green-yellow (1D), becoming to green-yellow (1C) and powdery while sporulating. Colony reverse green-yellow (1A), white at the margin.

*Additional specimens examined.* THAILAND, Chanthaburi Province, Khao Soi Dao District, Khao Soi Dao Wildlife Sanctuary, 13.10°N, 102.19°E, on stink bug, 1 Jan. 2000, *B. Thongnuch*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai*, *S. Mongkolsamrit*, *W. Chaygate* (BBH14343:BCC18114); Kanchanaburi Province, Thong Pha Phum District, Thung Yai Naresuan Wildlife Sanctuary, Krathon Ruesi Nature Trail, 15.33°N, 98.92°E, on moth adult, 2 Dec. 2005, *B. Thongnuch*, *K. Tasanathai*, *P. Srikitikulchai*, *W. Chaygate* (BBH15191:BCC19745); Phetchaburi Province, Kaeng Krachan District, Kaeng Krachan National Park, Ban Krang Camp Nature Trail, 12.88°N, 99.63°E, on blue milkweed beetle, 26 Apr. 2006, *B. Thongnuch*, *K. Tasanathai*, *R. Ridkaew*, *W. Chaygate* (BBH18375:BCC21293); Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Bueng Phai Nature Trail, 14.44°N, 101.37°E, on wasp, 29 Nov. 2006, *B. Thongnuch*, *P. Puyngain*, *T. Keokene*, *W. Chaygate* (BBH23082:BCC23823); Chiang Mai Province, Doi Inthanon National Park, Mae Chaem District, Mae Chaem Junction (KM.38) Nature Trail, 18.54°N, 98.52°E, on a grasshopper, 27 Nov. 2008, *A. Khonsanit*, *K. Tasanathai*, *P. Srikitikulchai* (BBH25297:BCC34350).

*Notes* — *Beauveria namnaoensis* is hardly distinguishable morphologically from other closely related species such as, *B. bassiana* and *B. neobassiana* (Table 2 and refer to the results on morphological variation). However, this new species is based on phylogenetic studies.

***Beauveria neobassiana*** Khons., Kobmoo & Luangsa-ard, *sp. nov.* — MycoBank MB No. 838939; Fig. 6

*Etymology.* The name is derived from the morphological similarity to *Beauveria bassiana*

*Holotypus*. THAILAND, Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Trail from Heo Sawat to Heo Sai Waterfall Nature Trail, 14.44°N, 101.37°E, on cicada adult, 20 May. 1996, K. Tasanathai, N.L. Hywel-Jones, S. Sivichai, S. Thienhirun (holotype BBH5054: ex-holotype strain BCC1848);

*Sexual morph* — Unknown.

*Asexual morph* — Specimens found on ant, adult beetles, adult cicadas, lepidopteran larvae, queen ants, moth and weevils. Hosts covered with white mycelium, powdery while sporulating. *Phialides* hyaline, solitary, smooth-walled, base subspherical, ovoid, (2)2.3–3.1(3.5) × (2)2.1–2.9(3) µm ( $n = 150$ ). *Conidia* hyaline, smooth-walled, globose, occasionally subglobose, (2)2–2.6(3) × (1.5)1.8–2.2(2.5) µm ( $n = 150$ ).

*Colony characteristics* — Colonies on PDA at room temperature attaining a diam of 20–21 mm in 10 d, 40–41 mm in 20 d, surface mycelium dense, floccose. Sporulation starts at 5 d after inoculation, white with green-yellow (2D), powdery when sporulating. Colony reverse yellow (6C), becoming to yellow-orange (20A) and white at the margin.

*Additional specimens examined*. THAILAND, Kanchanaburi Province, Sangkhla Buri District, Khao Laem National Park, Sum Nuk Buhd To, 15.02°N, 98.60°E, on beetle adult (*Coleoptera*), 21 Jun. 1995, N.L. Hywel-Jones, R. Nasit, S. Sivichai (BBH4614:BCC1446); Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Trail from Heo Sawat to Heo Sai Waterfall Nature Trail, 14.44°N, 101.37°E, on adult cicada, 20 May. 1996, K. Tasanathai, N.L. Hywel-Jones, S. Sivichai, S. Thienhirun (BBH5054:BCC1848); Phetchaburi Province, Kaeng Krachan District, Kaeng Krachan National Park, Khlong 1 Nature Trail, 12.88°N, 99.63°E, on moth, J.J. Luangsa-ard, K. Tasanathai, N.L. Hywel-Jones, P. Srikitikulchai, R. Nasit, S. Mongkolsamrit, W. Chaygate (BBH14301), Chiang Mai Province, Mae Chaem District, Doi Inthanon National Park, 18.54°N, 98.52°E; on ant, 1 Jan. 2000, B. Thongnuch, K. Tasanathai, P. Srikitikulchai, R. Ridkaew, S. Mongkolsamrit, W. Chaygate (BBH14373:BCC18124, BBH14374:BCC18171); Phetchabun Province,

465 Nam Nao District, Nam Nao National Park, Trail to nature study area, 16.74°N,  
 466 101.57°E, on weevil (*Coleoptera:Curculionoidea*), 23 May. 2000, K. *Tasanathai*, P.  
 467 *Lutthisungneon*, R. *Nasit*, S. *Sivichai* (BBH7666:BCC2660); Chiang Mai Province,  
 468 Fang District, Doi Phahompok National Park, Doi Phahompok Nature Trail, 20.00°N,  
 469 99.14°E, on queen ant, 5 Feb. 2006, B. *Thongnuch*, K. *Tasanathai*, S. *Mongkolsamrit*,  
 470 W. *Chaygate* (BBH16599:BCC20197); Nakhon Ratchasima Province, Pak Chong  
 471 District, Khao Yai National Park, Kong Kaeo Waterfall Nature Trail, 14.44°N,  
 472 101.37°E, on adult beetle, 12 Jun. 2007, C. *Chuaseeharonnachai*, S. *Sivichai*, S.  
 473 *Mongkolsamrit* (BBH24543:BCC25950); Chanthaburi Province, Soi Dao District,  
 474 Khao Soi Dao Wildlife Sanctuary, Headquater Nature Trail, 13.10°N, 102.19°E, on  
 475 adult cicada, 22 Jul. 2008, A. *Khonsanit*, J.J. *Luangsa-ard*, K. *Tasanathai*, P.  
 476 *Srikitikulchai*, S. *Mongkolsamrit* (BBH24402:BCC30545), on adult beetle  
 477 (*Coleoptera*), 22 Jul. 2008, A. *Khonsanit*, J.J. *Luangsa-ard*, K. *Tasanathai*, P.  
 478 *Srikitikulchai*, S. *Mongkolsamrit* (BBH23856:BCC31604); Tak Province, Umphang  
 479 District, Umphang Wildlife Sanctuary, Pi Tu Kro Waterfall Nature Trail, 15.92°N,  
 480 98.76°E, on queen ant, 26 Jun. 2001, A. *Khonsanit*, B. *Thongnuch*, J.J. *Luangsa-ard*,  
 481 K. *Tasanathai*, P. *Srikitikulchai*, S. *Mongkolsamrit*, W. *Chaygate*  
 482 (BBH23919:BCC31619); Chiang Rai Province, Chiang Khong District, Phlu Kaeng  
 483 Waterfall Nature Trail, 20.26°N, 100.39°E, on lepidopteran larva, 17 Jan. 2009, A.  
 484 *Khonsanit*, K. *Tasanathai*, P. *Srikitikulchai*, S. *Mongkolsamrit* (BBH26635:BCC35935);  
 485 Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Mo Sing  
 486 To Nature Trail, 14.44°N, 101.37°E, on adult beetle (*Coleoptera*), 20 Jul. 2009, K.  
 487 *Tasanathai*, P. *Srikitikulchai*, R. *Ridkaew*, S. *Mongkolsamrit*, T. *Chohmee*  
 488 (BBH26358:BCC37372); *ibid.*, 16 Aug. 2009, K. *Tasanathai*, P. *Srikitikulchai*, R.  
 489 *Ridkaew*, S. *Mongkolsamrit*, T. *Chohmee* (BBH26797:BCC37938); Chanthaburi  
 490 Province, Pong Nam Ron District, Suchin's Orchard, 12.92°N, 102.39°E, on adult  
 491 beetle (*Coleoptera*), 16 Dec. 2014, A. *Khonsanit*, D. *Thanakitpipattana*, N.  
 492 *Wiriyathanawudhiwong*, W. *Noisripoom* (BBH39718:BCC76579).

493

494 Notes — *Beauveria neobassiana* is hardly distinguishable morphologically from other  
 495 closely related species, such as *B. bassiana* and *B. namnaoensis* (Table 2 and refer  
 496 to the results on morphological variation). However, this new species is based on  
 497 phylogenetic studies.

498



499 ***Beauveria thailandica*** Khons., Kobmoo & Luangsa-ard, *sp. nov.* —MycoBank MB  
 500 838941; Fig. 6

501

502 *Etymology.* The epithet refers to the locality where the type specimen was found,  
 503 Thailand.

504

505 *Holotype.* THAILAND, Chanthaburi Province, Soi Dao District, Khao Soi Dao  
 506 Wildlife Sanctuary, 13.10°N, 102.19°E, on *Coleoptera* larva, 1 Jan. 2000, N. L.  
 507 Hywel-Jones (holotype BBH13831; ex-type culture BCC16585, TBRC8350).

508

509 *Sexual morph* — *Stromata* arising from the posterior part of coleopteran larva and  
 510 between head and thorax of click beetle, cylindrical with rounded apices, yellow  
 511 (8B–8A) when fresh and yellow-orange (19B–19A) when dry, 38–50 × 0.8–2.5 mm.  
 512 *Perithecia* semi-immersed, ovoid, (420)469.6–539(580) × (150)182.2–258.5(290) µm  
 513 ( $n = 30$ ). *Asci* hyaline, cylindrical, capitate, (180)–215.4–299.5(335) × (3)3.2–4.7(6) ×  
 514 µm ( $n = 50$ ). *Asci-caps* hyaline, hemispherical, (2)2.2–3.1(3) × (3.5)3.6–4.1(4) µm ( $n$   
 515 = 30). *Ascospores* hyaline, filiform, multiseptate, 241–320 × 1 µm ( $n = 8$ ). *Part-spores*  
 516 hyaline, cylindrical with truncated end, (4)–5.2–12.9(23) × 1 µm ( $n = 50$ ).

517

518 *Asexual morph* — Specimens found on adult cicada, earwigs, ladybugs, leafhoppers,  
 519 longhorn beetle, queen ant, stink bug and wasps. Hosts covered with white mycelium,  
 520 powdery when sporulating. *Phialides* hyaline, solitary, smooth-walled, base  
 521 subspherical to lageniform, ampulliform, mucronate, rostrate, (2)2.5–3.7(5) ×  
 522 (1.5)2–3(4) µm ( $n = 150$ ). *Conidia* hyaline, smooth-walled, obovoid, ovoid,  
 523 occasionally globose to subglobose, (2.5)3.1–4.3(6) × (1.5)1.6–2.3(3) µm ( $n = 150$ ).

524

525 *Colony characteristics* — Colony growth at room temperature attaining a diam of  
 526 21–22 mm 10 d, 30–32 mm in 20 d. Surface mycelium dense, convex to the agar  
 527 surface, floccose, cottony, sporulation starts at 10 d after inoculation, white to green-  
 528 yellow (1C). Colony reverse green-yellow (1C), white at the margin.

529

530 *Additional specimens examined.* THAILAND, Phetchabun Province, Nam Nao  
 531 District, Nam Nao National Park, Lum Nam Cheun Nature Trail, 16.74°N, 101.57°E,  
 532 on stink bug (*Hemiptera: Podopidae*), 11 Oct. 1994. *N.L. Hywel-Jones, R. Nasit, S.*  
 533 *Sivichai* (BBH4362:BCC1442); Phetchaburi Province, Kaeng Krachan District, Kaeng  
 534 Krachan National Park, KM. 15 on road to Tor Tip Waterfall, 12.88°N, 99.63°E, on  
 535 adult cicada (*Hemiptera: Cicadidae*), 23 May 1995, *N.L. Hywel-Jones*  
 536 (BBH4541:BCC1654); *ibid.*, on adult beetle (*Coleoptera*), 23 May 1995, *N.L. Hywel-*  
 537 *Jones* (BBH4542:BCC1655); *ibid.*, on earwigs (*Dermaptera: Chelisochidae*), 25 May  
 538 1995, *N.L. Hywel-Jones* (BBH4563:BCC2044); Kanchanaburi Province, Sangkhla  
 539 Buri District, Khao Laem National Park, Sum Nuk Buhd To, 15.02°N, 98.60°E, on  
 540 adult beetle (*Coleoptera*), 21 Jun. 1995, *N.L. Hywel-Jones, R. Nasit, S. Sivichai*  
 541 (BBH4561:BCC1665); Chanthaburi Province, Khao Soi Dao District, Khao Soi  
 542 Dao Wildlife Sanctuary, Behind office Nature Trail, 13.10°N, 102.19°E, on *Lepidoptera*  
 543 larva, 20 Jun. 1996, *K. Tasanathai, R. Nasit, S. Sivichai* (BBH5182:BCC1906);  
 544 Phetchabun Province, Nam Nao District, Nam Nao National Park, behind office to  
 545 bungalow 1 Nature Trail, 16.74°N, 101.57°E, on *Coleoptera* larva, 20 Aug. 1996, *K.*  
 546 *Tasanathai, R. Nasit, S. Sivichai* (BBH5227:BCC2086); Surat Thani Province,  
 547 Phanom District, Khao Sok National Park, Sip Et Shin Waterfall Nature Trail, 8.99°N,  
 548 98.63°E, on adult beetle (*Coleoptera*), *K. Tasanathai, N.L. Hywel-Jones, S. Sivichai*  
 549 (BBH5353:BCC2120); Phetchaburi Province, Kaeng Krachan District, Kaeng  
 550 Krachan National Park, KM. 15 on road to Tor Tip Waterfall, 12.88°N, 99.63°E, on  
 551 weevil bug (*Coleoptera: Curculionidae*), 2 Jun. 2000, *N.L. Hywel-Jones*  
 552 (BBH7769:BCC2676); Mae Hong Son Province, Mae Hong Son Road marker KM.  
 553 5.7, 19.30°N, 97.97°E, on Earwigs (*Dermaptera: Chelisochidae*), 30 Jun. 2002, *N.L.*  
 554 *Hywel-Jones, R. Nasit*, (BBH16766:BCC12907); Chanthaburi Province, Khao Soi  
 555 Dao District, Khao Soi Dao Wildlife Sanctuary, 13.10°N, 102.19°E, on longhorn beetle  
 556 (*Coleoptera*), 21 Aug. 2009, *B. Thongnuch, J.J. Luangsa-ard, K. Tasanathai, P.*  
 557 *Srikitikulchai, S. Mongkolsamrit, W. Chaygate* (BBH14344:BCC18115); Phetchabun  
 558 Province, Nam Nao District, Nam Nao National Park, Headquater Nature Trail,  
 559 16.74°N, 101.57°E, on adult leafhoppers (*Hemiptera, Cicadellidae*), 30 Jun. 2004, *B.*  
 560 *Thongnuch, K. Tasanathai, W. Chaygate* (BBH10140:BCC16183); Tak Province,  
 561 Umphang District, Umphang Wildlife Sanctuary, Pi Tu Kro Waterfall (Preto Lo Su),  
 562 15.92°N, 98.76°E, on adult longhorn beetle (*Coleoptera: Cerambycidae*), 26 June  
 563 2008, *A. Khonsanit, J.J. Luangsa-ard, K. Tasanathai, P. Srikitikulchai, S.*

564 *Mongkolsamrit* (BBH23918:BCC31618); Nakhon Ratchasima Province, Pak Chong  
 565 District, Khao Yai National Park, Mo Sing To Nature Trail, 14.7116666666667 N,  
 566 101.421666666667 E, on adult click beetle (*Coleoptera:Elateridae*), 18 Jun. 2009,  
 567 N.L. Hywel-Jones, K. Tasanathai, R. Ridkaew, S. Mongkolsamrit, T. Chohmee  
 568 (BBH38825:BCC36657); Nakhon Ratchasima Province, Pak Chong District, Khao  
 569 Yai National Park, Mo Sing To Nature Trail, 14.44°N, 101.37°E, on *Coleoptera* larva,  
 570 27 Sep. 2011, A. Khonsanit, K. Sansatchanon, K. Tasanathai, S. Mongkolsamrit, W.  
 571 Noisripoom (BBH32175: BCC49762); Chiang Mai Province, Chiang Dao District,  
 572 Chiang Dao Wildlife Sanctuary, Chiang Dao Wildlife Research Station, 19.39°N,  
 573 98.84°E, on *Lepidoptera* larva, 5 Oct. 2012, A. Khonsanit, K. Tasanathai, P.  
 574 Srikitikulchai, R. Promharn, W. Noisripoom (BBH38847:BCC56283); Chiang Mai  
 575 Province, Chiang Dao District, Ban Hua Thung Community Forest, 19.39°N, 98.84°E,  
 576 on adult wasp, 31 Oct. 2014, A. Khonsanit, D. Thanakitpipattana, K. Tasanathai, P.  
 577 Srikitikulchai, S. Wongkanoun, W. Noisripoom (BBH40622:BCC76509).

578

579 Notes — Phylogenetically, *B. thailandica* is closely related to *B. asiatica*. It occurs on  
 580 a wide range of insect hosts. Morphologically, *B. thailandica* occasionally has smaller  
 581 perithecia, shorter asci and ascospores as well as longer part-spores than *B. asiatica*  
 582 (Table 3). Detailed statistical analyses showed a significant difference in terms of  
 583 conidia length and a notable higher variation for the length and the width of phialides  
 584 and conidia.

585 **Table 2** Morphological comparisons of *Beauveria bassiana* species complex

Species	Host/substrate	Phialide (µm)	Conidia (µm)	Distribution	References
<i>B. bassiana</i> s.s.	Beetle adults, cucurbit beetle, mantid, maple leafcutter moth larva, moth adult, mulberry moth pupa, spittle bug, weevils	2.5–6 × 3–6	2–3 × 2–3	Brazil, Canada, Hungary, Morocco, Republic of Korea, Thailand, United States of America	Rehner et al. (2011), Khonsanit et al. 2020, This study
<i>B. namnaensis</i> sp. nov.	Blue milkweed beetle, grasshopper, moth, lepidopteran pupa, stink bug, wasp	2–5 × 1.5–3.5	1.5–4 × 1.5–3	Thailand	This study
<i>B. neobassiana</i> sp. nov.	Ant, beetle adults, cicada adults, lepidopteran larva, queen ants, moth, weevils	2–3.5 × 2–3	2–3 × 1.5–2.5	Thailand	This study

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597 **Table 3** Morphological comparisons of *Beauveria asiatica* species complex

Species	Host	Stromata (mm)	Perithecia (µm)	Asci (µm)	Asci-caps (µm)	Ascospores (µm)	Part-spores (µm)	Phialide (µm)	Conidia (µm)	Distribution	References
<i>B. asiatica</i> s.s.	Beetle adult, cicada adult, click beetle, dark black chafer beetle, grasshopper eggs, longhorn beetles	27–50 × 1.5–5	300–630 × 130–330	268–513 × 3–5	2–3 × 3–5	228–455 × 1	3–12 × 1	2.5–6.3 × 3–6	2.5–4.3 × 2–3	China, Republic of Korea, Thailand	Khonsanit et al. 2020, Rehner et al. 2011
<i>B. thailandica</i> sp. nov.	Cicada adult, click beetle, earwigs, ladybugs, leafhoppers, longhorn beetle, queen ant, stink bug, wasps	38–46 × 0.8–2.5	420–580 × 150–290	180–335 × 3–6	2–3 × 3.5–4	241–320 × 1	4–23 × 1	2–5 × 1.5–4	2.5–6 × 1.5–3	Thailand	This study

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## 600 Discussion

601 *Beauveria* is a genus with many cryptic species. There has been no comprehensive  
602 with genetic, morphological and chemical data. In this study, our objectives were to assess  
603 the species status of genetic groups found within *B. asiatica* sensu lato and *B. bassiana* s.l.  
604 based on population genomics, morphological and chemical data.

### 606 **Phylogenetics Species Criterion**

607 The intraspecific genetic groups as found in these two species complexes by  
608 Khonsanit et al. (2020) were recovered by population genomics data in this study. The main  
609 question is whether they should be considered as distinct species. The evolutionary species  
610 concept (ESC) defined a species as “a single lineage of ancestor-descendant populations  
611 which maintain its identity from other such lineages, and which has its own evolutionary  
612 tendencies and historical fate” (Wiley 1978). This concept is not debated but is not an  
613 operational species delimitation criterion (De Queiroz 2007; Giraud et al. 2008). For fungi,  
614 the so-called phylogenetic species concept (PSC) is an operational concept that has been  
615 predominant and proposed as the most consistent to ESC (Taylor et al. 2000). According to  
616 the theoretical concept related to the most common ancestor and descending progenies,  
617 PSC is naturally associated to the monophyly and consistency of clades across genes,  
618 which became the basis to phylogenetic species recognition (PSR) (Cracraft 1983; Mishler  
619 & Brandon 1987; Mishler & Theriot 2000). According to this criterion, *B. asiatica* Clade C,  
620 Clade Mixed, and *B. bassiana* Clade A, Clade B and Clade C should be considered as  
621 distinct species. However, monophyly-based species recognition received also some  
622 criticisms; monophyly-based species recognition cannot apply to asexual species because,  
623 without recombination, all lineages are monophyletic and with consistent gene genealogies.  
624 *Beauveria* had actually long been thought to be an asexual genus (Basalmo-Crivelli 1835).  
625 Our analyses of LD decay suggested substantial within some of these clades, which is  
626 consistent to observations of teleomorphs for several *Beauveria* species, including *B.*  
627 *bassiana* (Khonsanit et al. 2020; Rehner et al. 2011). The phylogenetic networks which take  
628 into account reticulated evolution still showed clear separation between them.

629 However, PSC can be arbitrary in regard of the limit of where to put species  
630 boundaries as any kind of molecular markers with polymorphisms would allow grouping  
631 individuals into monophyletic clades based on their respective alleles. PSC was thus  
632 proposed to rely on the concordance between distinct gene genealogies (Avis & Ball; Baum  
633 & Shaw 1995), resulting in the genealogical concordance phylogenetic species recognition  
634 (GCPSR) criterion for which the limit of species is placed where there is a transition from the

concordance among branches connecting different species to the conflict between branches within species due to intraspecific recombination (Taylor et al. 2000). Some fungal species were in fact erroneously defined based on only concatenated analysis of multi-gene phylogenies without concordance between markers and any corroboration from morphological, chemical and ecological data (Liu et al. 2016). Our results showed that the different genetic clades had actually high concordance factors except for *B. asiatica* Clade Mixed. Except for this latest case, the clades as revealed by whole-genome data are thus supported as distinct phylogenetic species.

### ***Insights from the morphology, chemistry and host association***

Previous studies have shown that the size and the shape of conidia can be used, to some extent, to discriminate between species but these characters were largely overlapping across species in *Beauveria* (Rehner & Buckley 2005; Imoulan et al. 2017; Khonsanit et al. 2020). Our study revealed that the morphological differences between clades within *B. asiatica* and *B. bassiana* were much less evident than the genomic data. Nevertheless, the morphology was significantly different between *B. asiatica* Clade C and Clade Mixed (the length of conidia). The chemotaxonomy of *Beauveria* has not been updated from a few studies conducted more than three decades ago (Mugnai et al. 1989; Bridge et al. 1990), which revealed that chemical profiles corresponded more or less to species based on morphological criteria but were heterogenous and overlapping between populations within species (e.g. *B. bassiana*). In our study, we found a discriminant pattern of secondary metabolites distribution between *B. asiatica* and *B. bassiana*, but not between the clades within each species. Altogether, our findings support the idea that the two *Beauveria* species are composed of sibling species with similar phenotypes, i.e. cryptic species.

Our study also suggested ecological differentiation between these clades. *Beauveria* is known to display a very broad host range as a whole (Imoulan et al. 2017) but the host ranges of the various species had not been well established. By reconstructing from the information on the hosts' taxonomic order associated to our specimens, the most recent common ancestor (MRCA) of *Beauveria* seemed to be predominantly associated to *Coleoptera*. The MRCA of *B. asiatica* Clade Mixed had actually broader host range than Clade C, and the MRCA of *B. bassiana* Clade A had a wider host range than Clade B and C. These two particular clades seemed to have gained the ability to exploit other insect orders than *Coleoptera*. This might be accompanied by specific selection, that may have leaved genomic signatures which could be investigated in future studies. There are many

examples of pathogenic fungi showing notable genomic modification related to host range and specificity (Wang & Wang 2017; Borah et al. 2018).

## **Taxonomy**

Given the evidence discussed above, we propose *B. bassiana* Clade A and Clade B as new species, named as *B. namnaoensis* and *B. neobassiana*, while Clade C which includes the type strain ARSEF1564 can be considered as *B. bassiana* sensu stricto. Regarding *B. asiatica* sensu lato, the Clade Mixed is proposed here as a new species, *B. thailandica*, and the Clade C containing the type strain ARSEF4850 remains *B. asiatica* s. s. The description and photographic materials for the novel species are given above in the results. These species are largely overlapping in their distribution, e.g. sympatric species, (Fig.S4) but are genetically well isolated with limited recombination between them. Despite lack of distinctive distribution pattern of secondary metabolites, the genomic, morphological and ecological data supported that they are distinct species.

## **Future investigation**

Investigation regarding the sexual compatibility between the closely related species within each of the two species complexes should be conducted in the future to assess whether they could fit the criterion of reproductive isolation for biological species (De Queiroz 2005). Reproduction mode and mating systems are extremely variable in fungi, from pure clonality to outcrossing sexual reproduction, with significant implications in genetic diversity and adaptability (Billiard et al. 2011; Taylor et al. 2015). Some studies in fungal pathogens have already shown that closely related cryptic species could be intersterile (e.g. *Calonectria pauciramosa*: Lombard et al. 2010) or sexually compatible with post-mating isolation (e.g. *Microbotryum* spp.: de Vienne et al. 2009). *Beauveria* was initially proposed to be an anamorphic genus, i.e. being present naturally only under asexual form (Basalmo-Crivelli 1835). Sexual reproduction structures could however occasionally be observed in natural habitats (Sanjuan et al. 2014; Khonsanit et al. 2020). Our study furthermore suggested some extent of genetic recombination and sexual reproduction in all the newly proposed species.

## **Conclusion**

Our work showed that whole-genome data could provide strong support for intra-species genetic groups and be powerful for delimiting species. During the last decade, genomics data have greatly contributed to the elucidation of species complexes in many



organisms (Chan et al. 2017; Cerca et al. 2021) including fungi (Sepúlveda et al. 2017; Kobmoo et al. 2019; Matute & Sepúlveda 2019). For Hypocrealean entomopathogenic fungi, such approaches should be particularly useful as there are likely many cryptic species in different genera (*Blackwellomyces* and *Cordyceps*: Mongkolsamrit et al. 2020b), *Isaria*: Mongkolsamrit et al. 2018, *Metarhizium*: Mongkolsamrit et al. 2020). Many taxonomic works were based on a few samples with a limited number of markers which has likely led to the underestimation of the diversity in this fungal group. Combining whole-genome data, thorough morphological examination with statistical analyses and chemical profiling will contribute to establish a solid basis for species discovery in fungi.

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## 914 **Figures Captions**

915

916 **Fig.1** Population genomics analyses: a. A circular NJ tree based on whole-genome SNPs,  
 917 the red asterisks represent the type strains of *Beauveria asiatica* (ARSEF4850) and *B.*  
 918 *bassiana* (ARSEF1564), thick branches correspond to those supporting 100%  
 919 bootstrapp; b. Principal component analysis (PCA) for total sampling; c. Bayesian  
 920 clustering for total sampling; d. PCA for *B. asiatica*; e. PCA for *B. bassiana*; f. PCA for  
 921 *B. asiatica*.

922

923 **Fig.2A** Bayesian phylogenetic tree based on concatenated 1034 genes in *Beauveria* species.  
 924 The red dots represent nodes supported by 1.00 posterior probability. The highlighted  
 925 clades are supported by Bayesian concordance factors with 95% confidence interval.

926

927 **Fig.3** Morphometric analysis: a. Boxplots representing the distribution of the length and the  
 928 width of phialides and conidia between *Beauveria asiatica* and *B. bassiana*. The red  
 929 asterisks denote significant difference between the two species; b. Boxplots  
 930 representing the distribution of the length and the width of phialides and conidia  
 931 between the clades within *B. asiatica* (left panel) and within *B. bassiana* (right panel).

932

933 **Fig.4** Statistical analyses of secondary metabolite profiles resulting from liquid  
 934 chromatography-mass spectrometry (LC-MS): a. a Euclidean distance-based NJ tree,  
 935 b. Principal component analysis (PCA) for total sampling (top panel) and for the clades  
 936 within *Beauveria asiatica* and *B. bassiana* (bottom panel).

937

938 **Fig.5** Maximum-likelihood reconstruction of ancestral host association.

939

940 **Fig.6** *Beauveria namnaoensis* (a–g). a–c. Fungus on the hosts; d. colony obverse on PDA  
 941 after 10 d; e. colony obverse on PDA after 20 d; f. phialides and conidia; g. conidia.  
 942 *Beauveria neobassiana* (h–o). h–k. Fungus on the hosts; l. colony obverse on PDA  
 943 after 10 d; m. obverse on PDA after 20 d; n. phialides and conidia; o. conidia. *Beauveria*  
 944 *thailandica* (a–zc). p–t. Fungus on the hosts; u. fertile head; v. perithecia; w. asci with  
 945 asci caps; x. ascospore; y. part-spores; z. colony obverse on PDA after 10 d; za.  
 946 obverse on PDA after 20 d; zb. phialides and conidia; zc. conidia. — Scale bars: a, d,  
 947 e, i, l, m, p, z, za = 10 mm; b, c, h, i, j, k, q, r, s, t = 5 mm; u = 1 mm; v = 200 µm; x = 20  
 948 µm; f, n, w, y, zb = 10 µm; g, o, zc = 5 µm.

## Supplementary Data

**Fig.S1** Analyses of recombination footprint for *Beauveria asiatica* (a.) and *B. bassiana* (b.).

The left panel represents phylogenetic networks based on SNPs from respective species. The right panel represents the analyses of linkage disequilibrium (LD) decay.

**Fig.S2** Liquid chromatography-mass spectrometry (LC-MS) profiles of mycelia extracts from *Beauveria asiatica* and *B. bassiana* species complexes.

**Fig.S3** Liquid chromatography-mass spectrometry (LC-MS) profiles of mycelia extracts from *Beauveria spp.* included for comparison with *B. asiatica* and *B. bassiana*.

**Fig.S4** Distribution map of *Beauveria asiatica* sensu lato (left panel) and *B. bassiana* s.l. (right panel).

**Table S1** List of *Beauveria* strains used in the study. The species were attributed according to previous studies or routine identification in our laboratory. Specimens of *B. asiatica* were proposed to be a novel species, *B. thailandica*, while some of *B. bassiana* were proposed as new species, *B. neobassiana* or *B. namnaoensis* sp. nov., based on phylogenomics results.

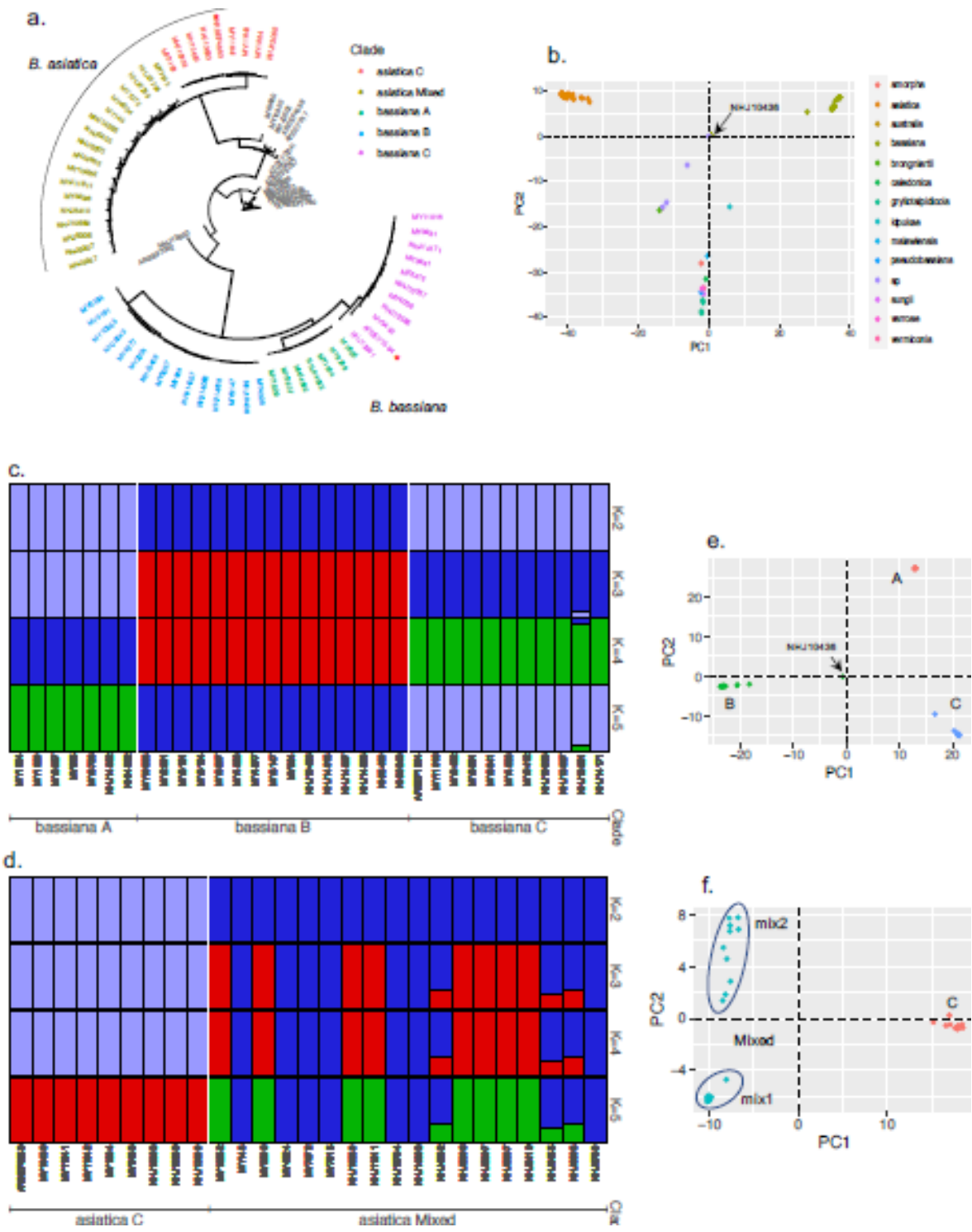
Note 1 : s.s. = sensu stricto, sp. nov. = species nouveau.

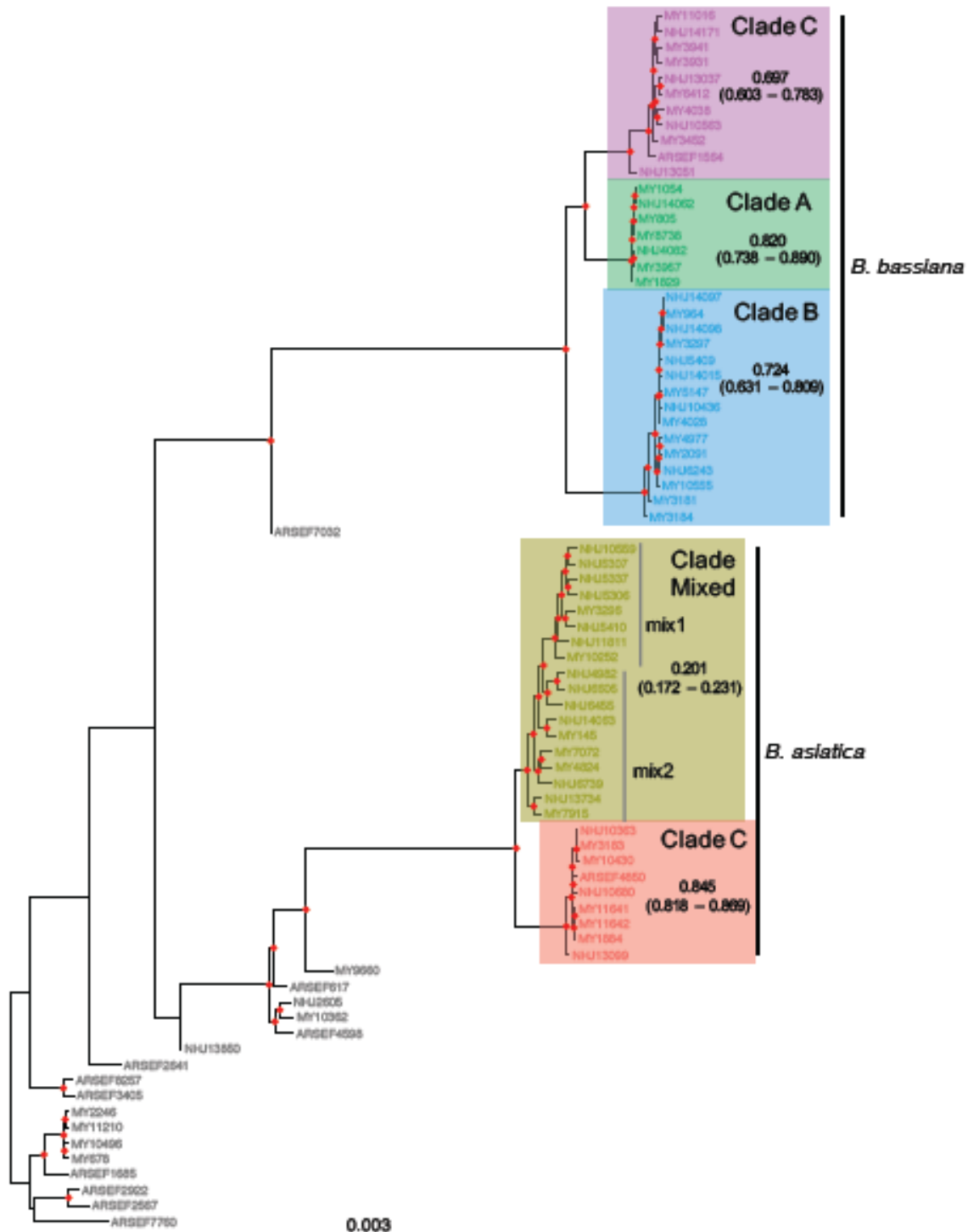
Note 2: <sup>a</sup> = used in analyses of secondary metabolites profiling

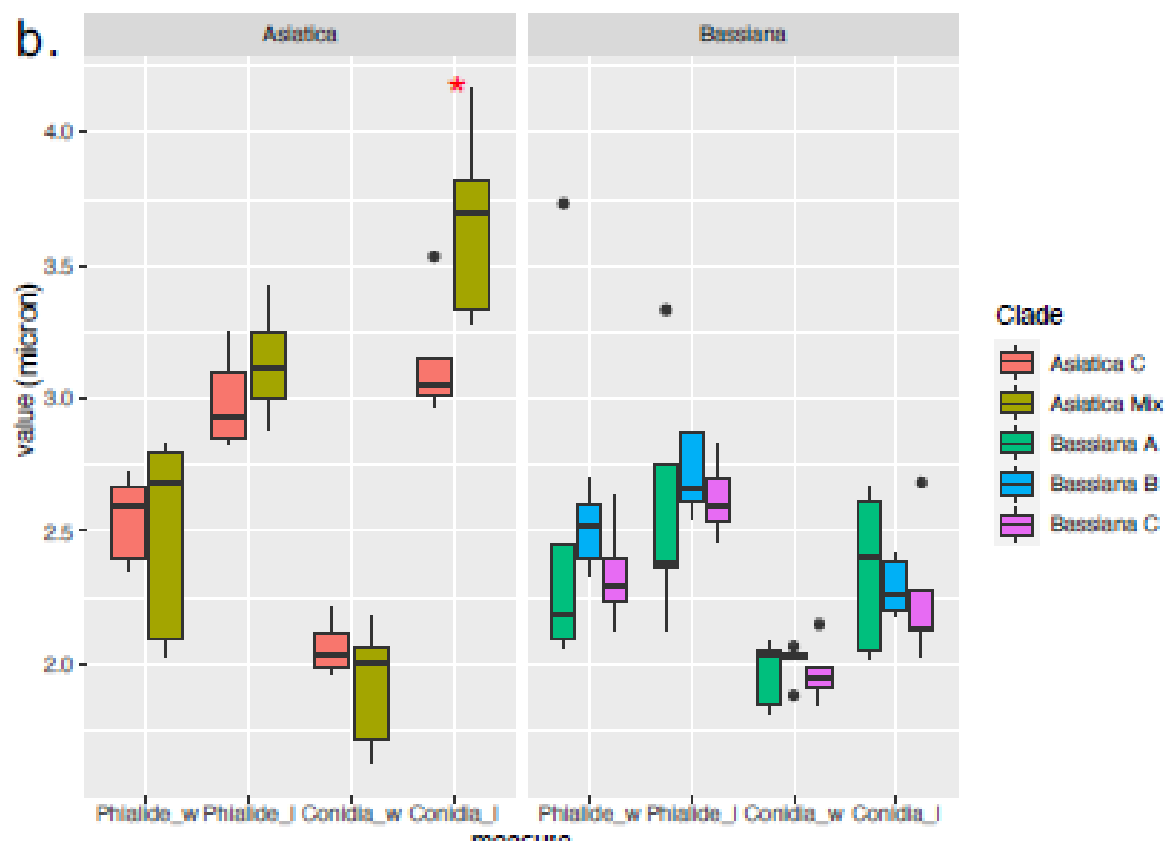
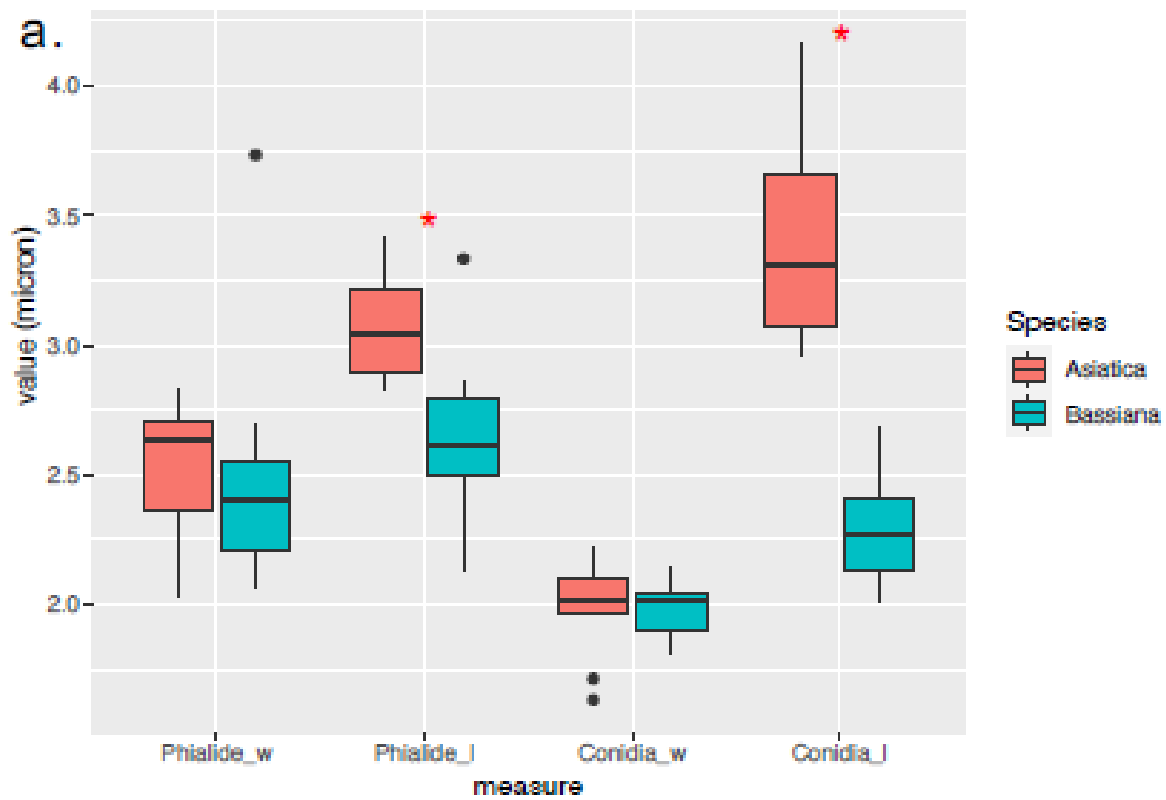
**Table S2** Average width and length (µm) of conidia and phialides per strain for the strains used in statistical analyses of morphological traits.

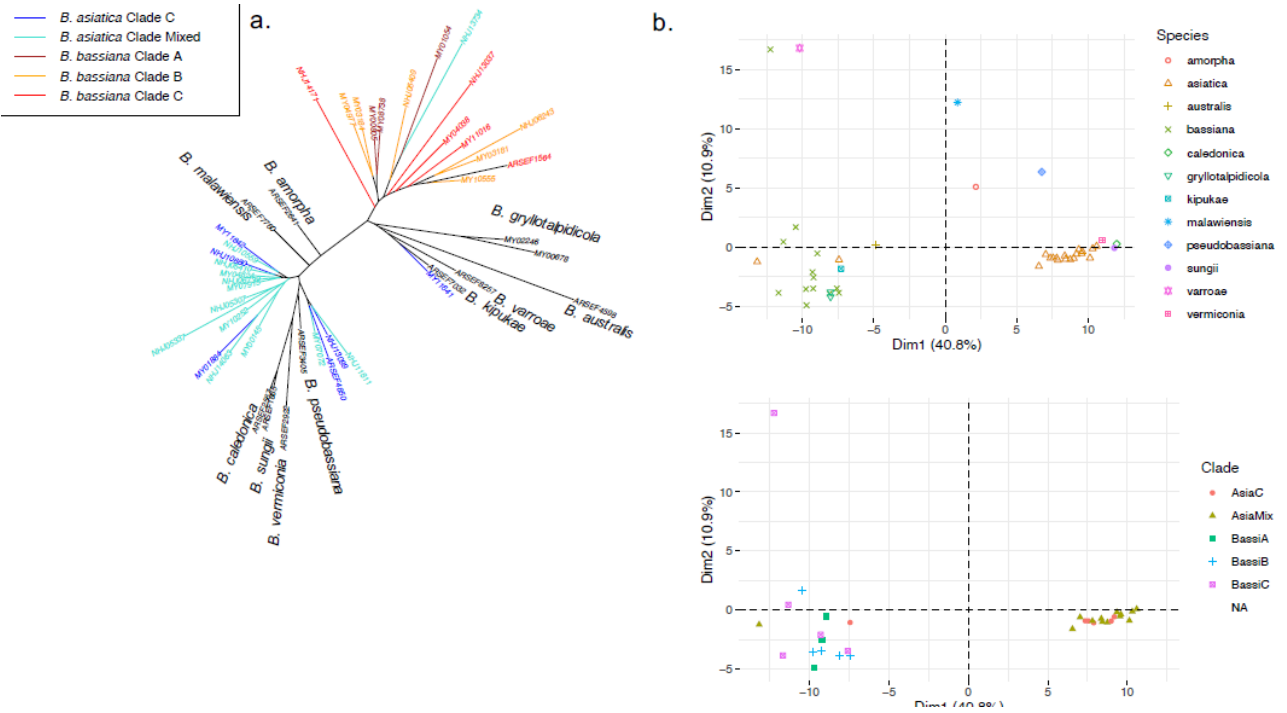
**Table S3** The results of statistical analyses to test difference between clades within *B. asiatica* and *B. bassiana*. \* denotes a statistical significance ( $p < 0.05$ ).

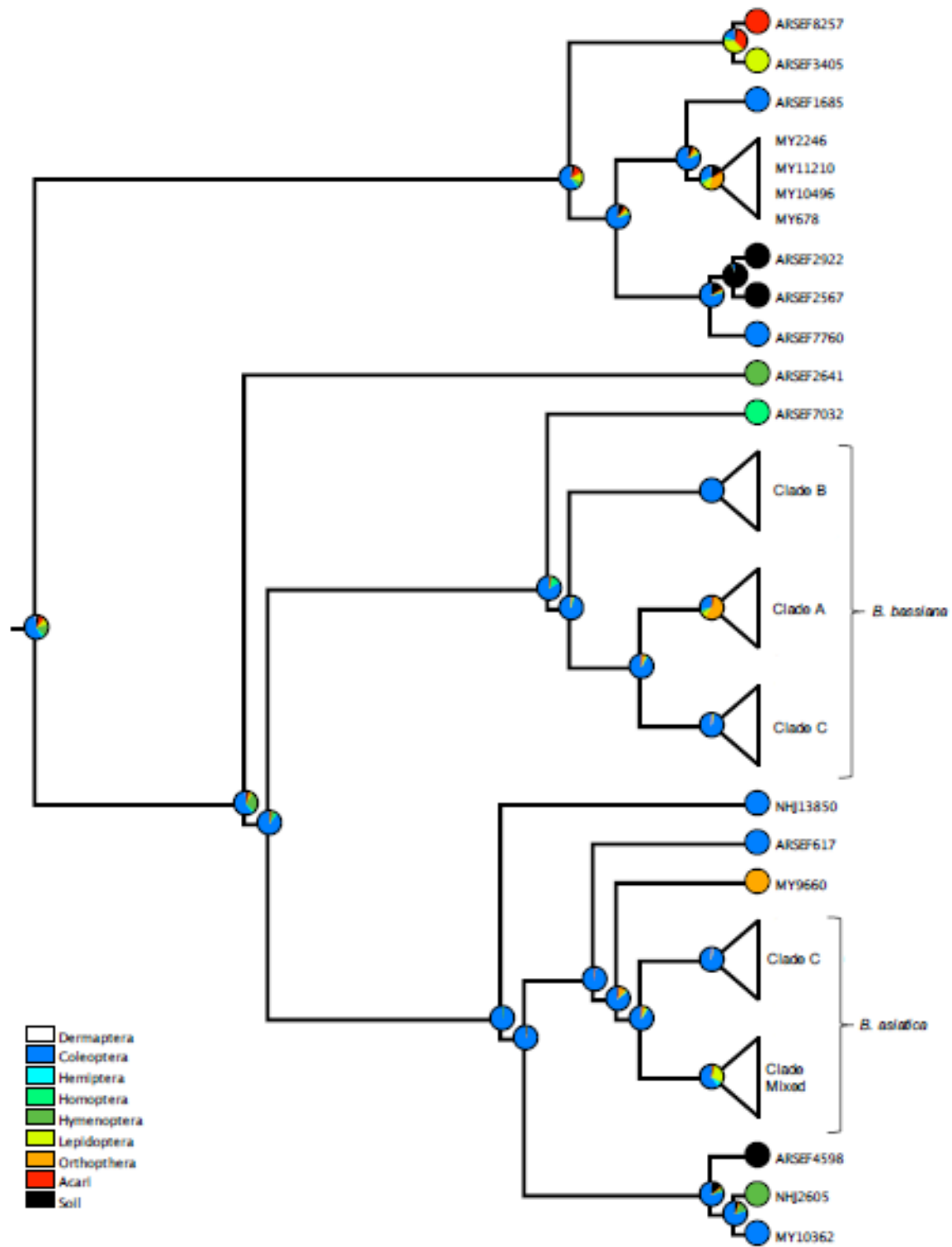


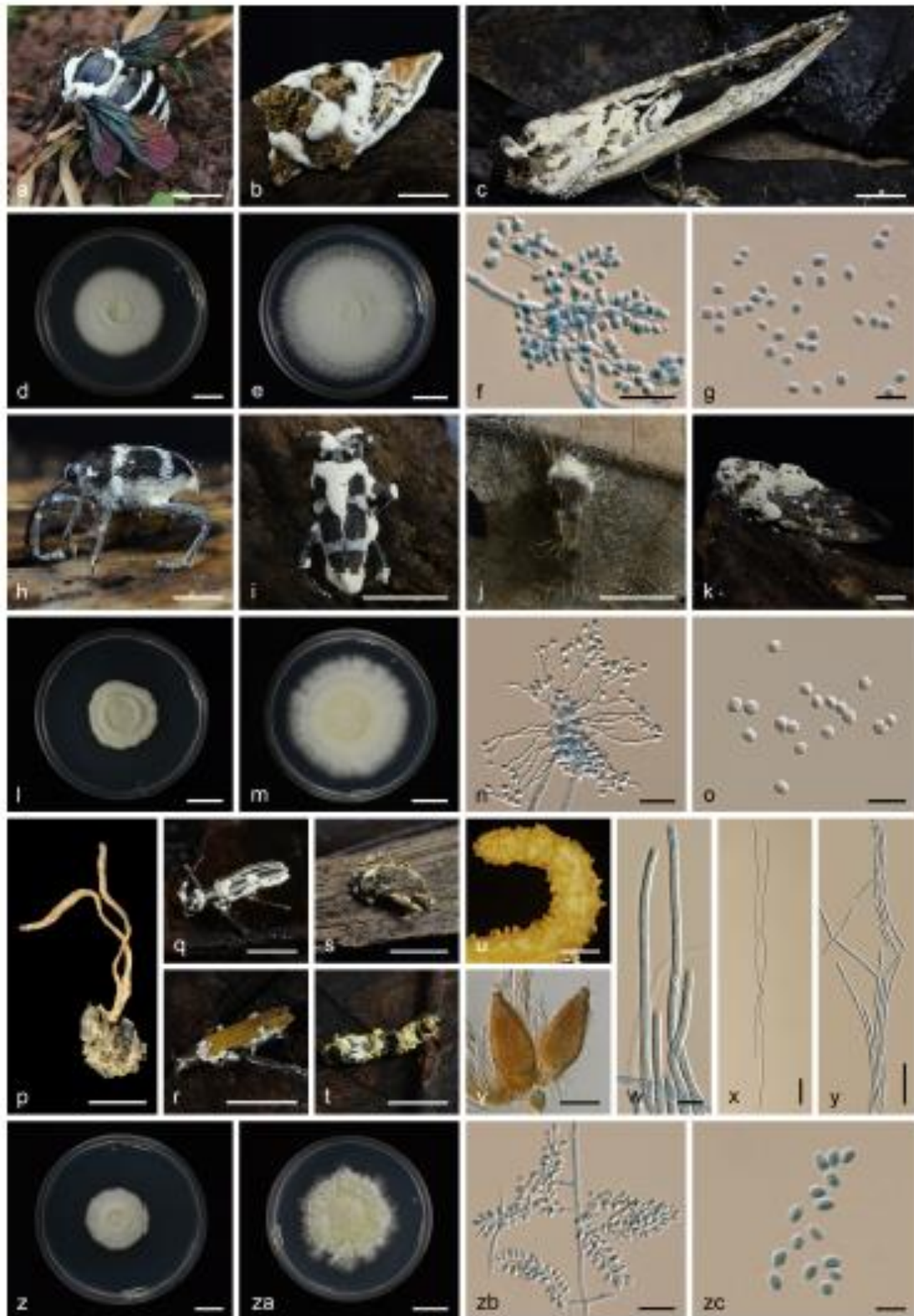












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