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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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Key words

Beauveria
chemotaxonomy
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taxonomy

Abstract Fungi are rich in complexes of cryptic species that need a combination of different approaches to be delimited, including genomic information. *Beauveria* (*Cordycipitaceae*, *Hypocreales*) is a well-known genus of entomopathogenic fungi, used as a biocontrol agent. In this study we present a polyphasic taxonomy regarding two widely distributed complexes of *Beauveria*: *B. asiatica* and *B. bassiana* s.lat. 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 among 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 among the clades within *B. bassiana* while conidial length was significantly different among clades within *B. asiatica*. The secondary metabolite profiles obtained by liquid chromatography-mass spectrometry (LC-MS) allowed a distinction between *B. asiatica* and *B. bassiana*, but not between the clades therein. Based on these genomic, morphological, chemical data, we proposed a clade of *B. asiatica* as a new species, named *B. thailandica*, and two clades of *B. bassiana* to respectively represent *B. namnaoensis* and *B. neobassiana* spp. nov. Such closely related but divergent species with different host ranges have potential to elucidate the evolution of host specificity, with potential biocontrol application.

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INTRODUCTION

Fungi are rich in complexes of cryptic species, i.e., morphologically similar but genetically isolated. In pathogenic fungi, cryptic 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 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 with biocontrol agents.

Beauveria bassiana was first discovered in the 18th century as representative of the genus *Botrytis* (Basalmo-Crivelli 1835), before being renamed as the first species of the genus *Beauveria* (Vuillemin 1912). It is distributed worldwide (Rehner & Buckley 2005, Imoulan et al. 2017). Being recognised as a rich source of efficient mycoinsecticides, in particular *B. bassiana* and *B. brongniartii* (Zimmermann 2007), the genus *Beauveria* has received a lot of attention as potential biological control agents (García-Estrada et al. 2016). The discovery of new species and their precise delimitation contribute to enlarge the potential for finding suitable biocontrol agents and for their safe use. *Beauveria* is characterised 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 distantly related ones (Khonsanit et al. 2020). Furthermore, many new *Beauveria* species have been proposed in recent years, 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 waiting to be elucidated. However, there has never been a thorough morphometric analysis with solid statistical tests.

Although species of *Beauveria* are known to produce an array of secondary metabolites (Xu et al. 2008, 2009, Rohlfs & 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 several years ago for *Beauveria* (see Mugnai et al. 1989, Bridge et al. 1990) without any update for recently discovered species. Chemical compounds allowed the distinction between popula-

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tions within *B. bassiana* (Bridge et al. 1990) but this finding was not compared with genetic data.

The abundant genomic resources presently available 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 recently been used to reveal fungal cryptic species (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 a 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 these 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 *Beauveria*, 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 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 a multidisciplinary approach 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 thorough taxonomic revision should indeed be based on a combined dataset including sufficient 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 the BIOTEC culture collection (BCC) and ARS collection of entomopathogenic fungal cultures (ARSEF), including the ex-type strains of known species and strains previously identified as *B. asiatica* or *B. bassiana* 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 sterilised 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. (2019); 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 100 V for 1/2 h.

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 MGIEazy 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 v. 1.5.6.11 software package, raw reads were de-multiplexed 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 v. 1.5.6.11 alignment software (Minimap2 v. 2.11-r797-v03) and the variants were called using GATK HaplotypeCaller v. 3.8. SNP markers with poor quality data were filtered out using the following criteria:

– a minor allele frequency < 0.1;
– depth coverage less than 10 ×;
– 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 associated to the BioProject accession PRJNA744643. The SNPs data were deposited at Mendeley Data repository (Sonthirod et al. 2021).

Population structures, genetic diversity and linkage disequilibrium

The selected SNPs (729 549 SNPs) were aligned and subjected to a maximum-likelihood based phylogenetic tree inference using RaxML v. 8 (Stamatakis 2014), by specifying GTRCAT model with Lewis biases ascertainment correction. The reliability of the resulting tree was evaluated with 1 000 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 2008). 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. Neighbour-net phylogenetic network based on p-distance was constructed using the software SplitsTree v. 4.14.18 (Huson & Bryant 2006).

Species tree

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 (Katoh & Standley 2013). A total of 1 132 genes were first selected based on:

– their size (1 Kbp minimum and 10 Kbp maximum) to ensure an absence of recombination within genes;
– 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;
– 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 M 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 1 034 genes. The 1 034 genes sequences were also concatenated and subjected to MrBayes with the model GTRGAMMAI, as chosen by ModelTest-NG (Darriba et al. 2020), for 5 M of MCMC generations. The final tree was obtained after a 25 % burn-in phase.

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 1 034 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).

Secondary metabolites profiling

Strains of *Beauveria*, selected to represent the various genetic groups within *B. asiatica* and *B. bassiana* as well as ex-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 after glucose depletion for an individual strain was set to be half the time required for that particular strain to reach the glucose depletion (Table S2). 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.

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). A single extract per strain was obtained. 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 diam, 2.1 × 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 0–12 min, 12–95 % B; 12–14 min, 12–95 % B; 14–16 min, 12–95 % 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. Each extract was injected three times into the LC-MS as technical replicates.

The software Compound Discoverer v. 3.1 was used for data pre-processing steps. The MS raw files were subjected to peak alignment, peak picking, adduct grouping 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 5×10^5 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 neighbour-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).

Conservation of secondary metabolites gene clusters

We examined whether the distribution of secondary metabolites production among clades and species was reflected by the conservation pattern of secondary metabolites gene clusters (SMGCs). First, we inferred and annotated SMGCs present in the reference genome (ARSEF8028) using antiSMASH fungal v. 4.1.0 (Blin et al. 2017). The deduplicated and filtered mapped reads used in the phylogenomics above were proceeded to the pipeline of CNVnators (Abyzov et al. 2011) in order to detect regions of deletion in each isolate, based on normalised read depth. The detected blocks of deletion were intersected with the position of SMGCs and any SMGC with more than 50 % of its length overlapping a deletion is considered as absent or non-functional. The SMGCs presence/absence patterns were used to infer a cladogram representing the clustering of the isolates of *Beauveria* based on binary distances.

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 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 S3). The data were analysed 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 of *B. asiatica* corresponds to the Clade C as found in Khonsanit et al. (2020), including the ex-type strain ARSEF 4850; the other clade comprises a mix of individuals from the Clades A–D from that previous study, therefore named here as 'Clade Mixed'. The three clades from *B. bassiana* correspond to the Clades A–C established in this species by Khonsanit et al. (2020); the Clade C includes the ex-type strain ARSEF 1564. The principal component analysis (PCA) also showed clear

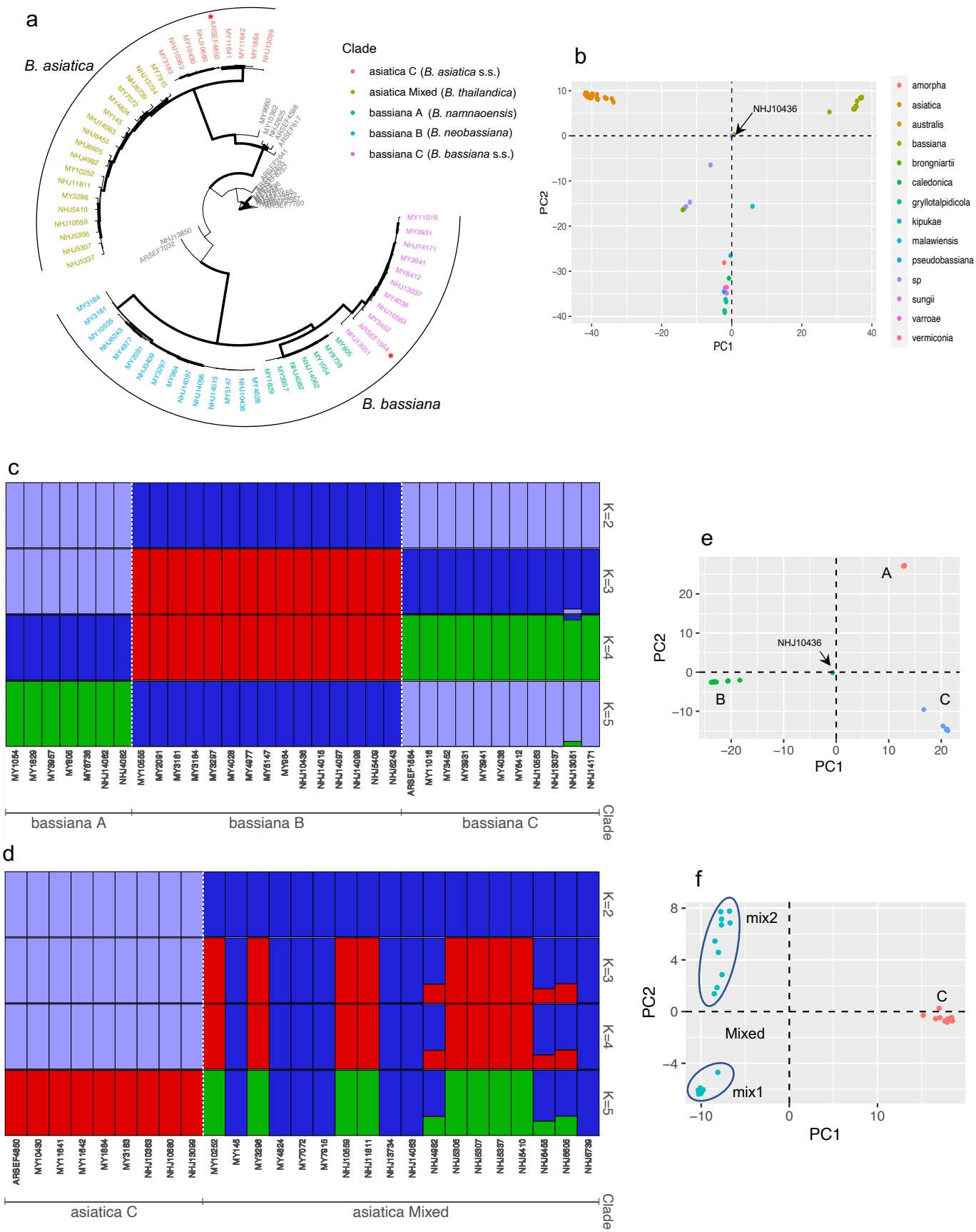

Fig. 1 Population genomics analyses. a. A circular NJ tree based on whole-genome SNPs, the red asterisks represent the type strains of *Beauveria asiatica* (ARSEF4850) and *B. bassiana* (ARSEF1564), thick branches correspond to those supporting 100 % bootstrap; b. principal component analysis (PCA) for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

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 (Pi: italic values forming the diagonal) within clades.

		B. asiatica		*B. bassiana*		
		Clade C	Clade Mixed	Clade A	Clade B	Clade C
B. asiatica	Clade C	*0.009*	0.0875	0.651	0.675	0.669
	Clade Mixed	0.742	*0.036*	0.630	0.654	0.648
B. bassiana	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*

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 from each other. The Bayesian clustering within each of the two species recovered the same genetic clusters as above (Fig. 1c–d) but, for *B. asiatica*, an additional subdivision was observed within Clade Mixed (Fig. 1d). 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 subclusters 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, e). 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 (Pi) 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),

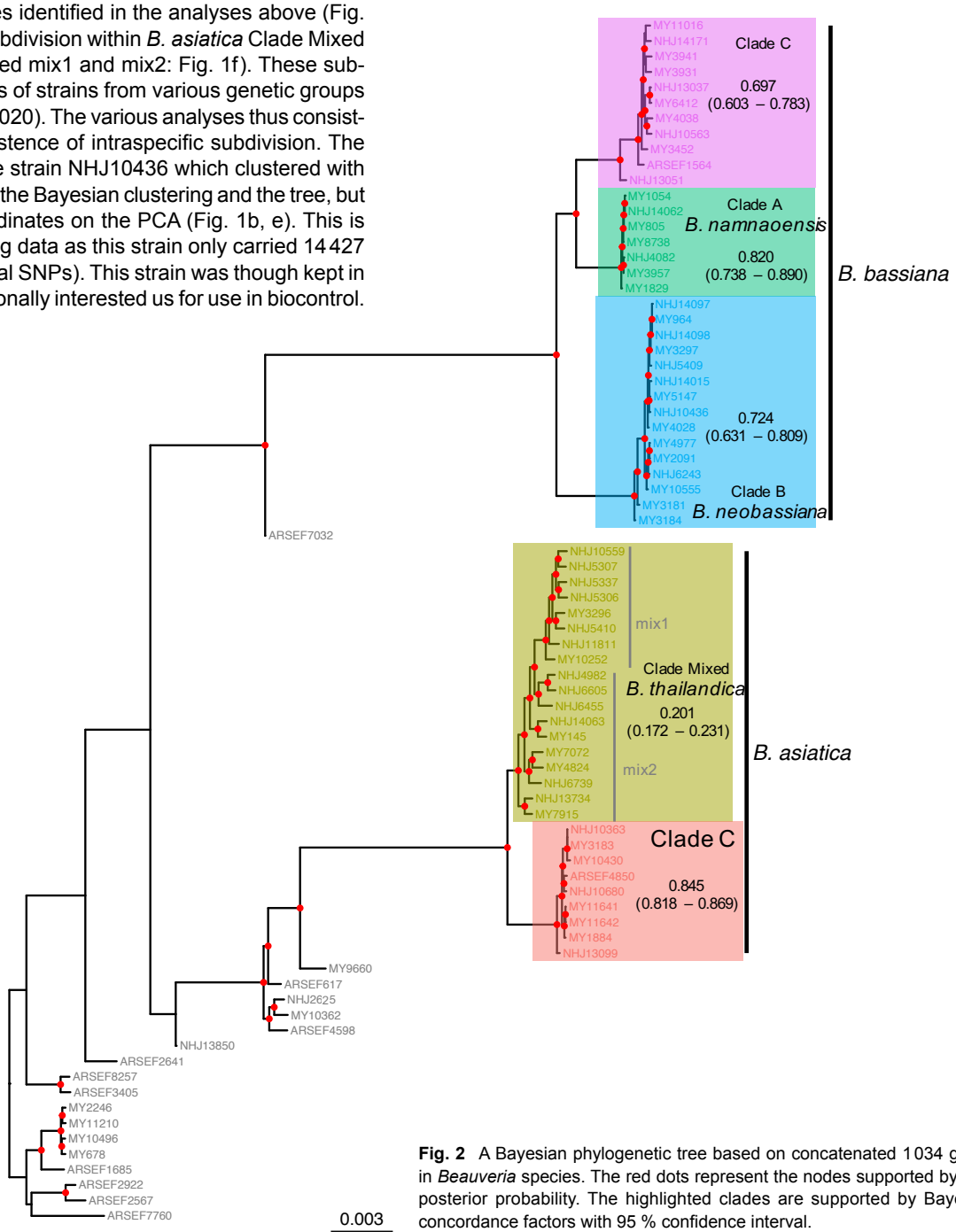

Fig. 2 A Bayesian phylogenetic tree based on concatenated 1034 genes in *Beauveria* species. The red dots represent the nodes supported by 1.00 posterior probability. The highlighted clades are supported by Bayesian concordance factors with 95 % confidence interval.

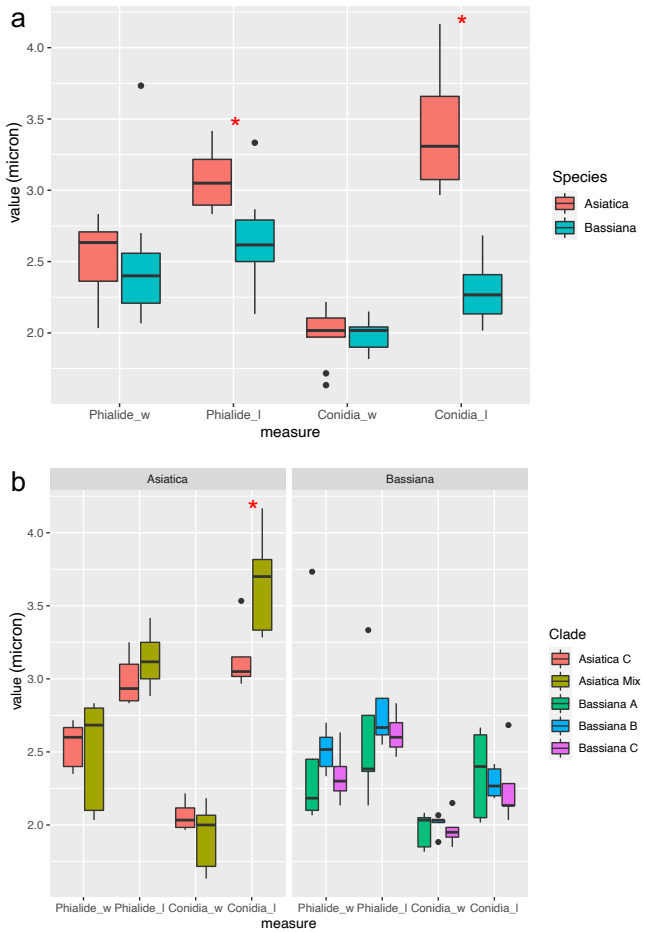

Fig. 3 Morphometric analysis. a. Boxplots representing the distribution of the length and the width of phialides and conidia between *Beauveria asiatica* and *B. bassiana*. The red asterisks denote significant difference between the two species; b. boxplots representing the distribution of the length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

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 (Pi) was higher in *B. asiatica* Clade Mixed (0.024 and 0.036 for the sub-clusters mix1 and mix2, respectively) than in Clade C (0.009). In *B. bassiana*, Clade B had the highest Pi (0.031), followed by Clade C (0.018) and Clade A (0.003).

Phylogenetic network and linkage disequilibrium

The Neighbour-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 1 034 concatenated genes recovered strongly supported monophyletic clades corresponding to the genetic subdivisions within *B. asiatica* (Clade C and Clade Mixed) and *B. bassiana* (Clades A–C) (Fig. 2). The sub-cluster mix 1 within *B. asiatica* Clade Mixed was recovered as a well-supported monophyletic clade but not the sub-cluster mix 2 which did not form a monophyletic clade, 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*

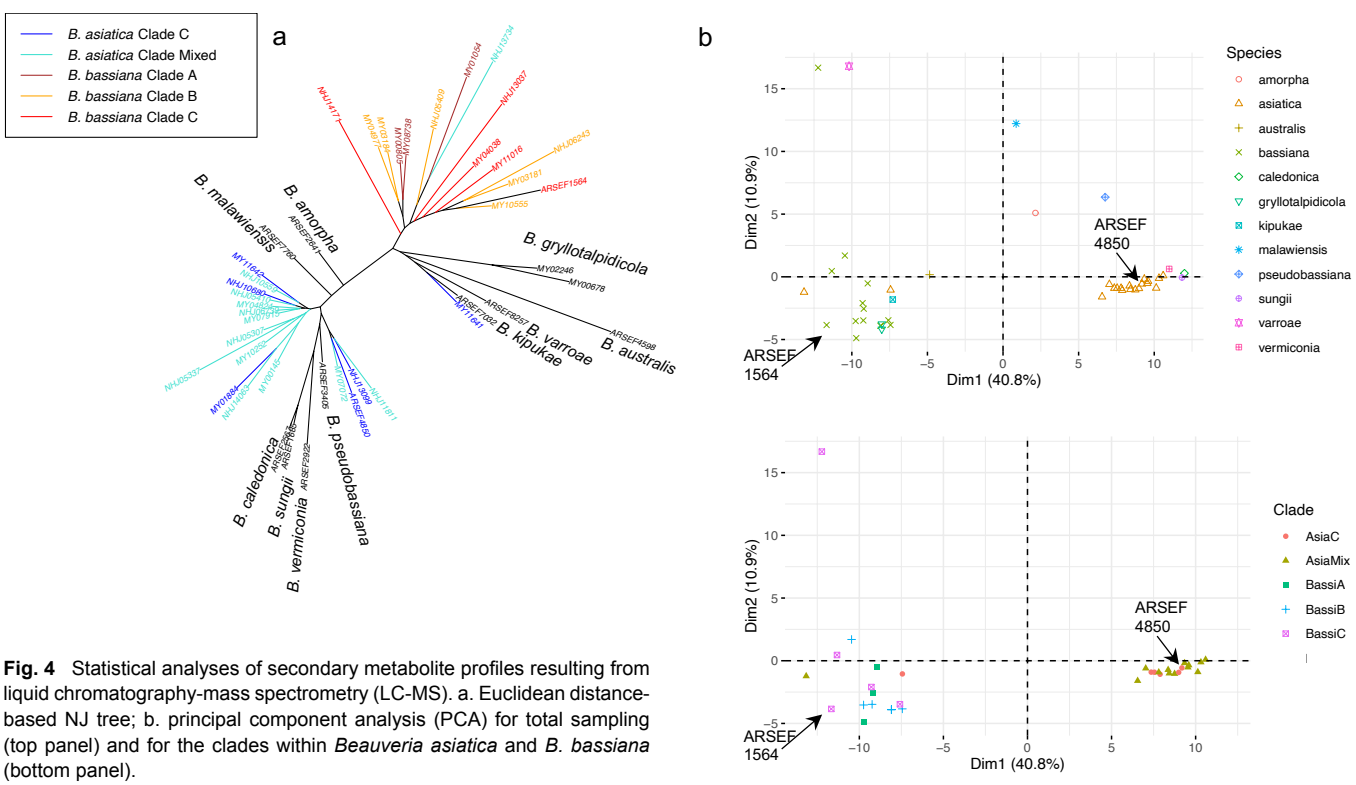

Fig. 4 Statistical analyses of secondary metabolite profiles resulting from liquid chromatography-mass spectrometry (LC-MS). a. Euclidean distance-based NJ tree; b. principal component analysis (PCA) for total sampling (top panel) and for the clades within *Beauveria asiatica* and *B. bassiana* (bottom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

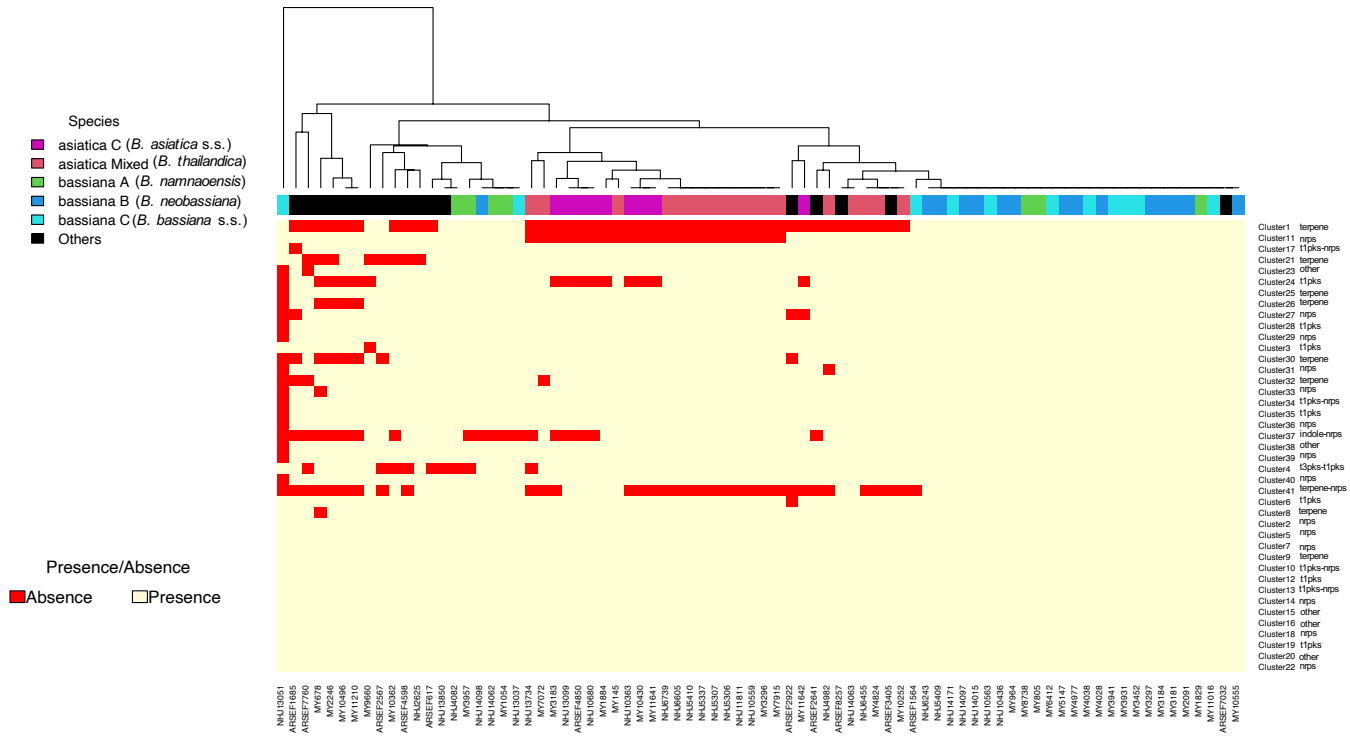

Fig. 5 The presence/absence of secondary metabolites gene clusters among *Beauveria* species. The isolates were ordered from left to right according to a binary distance-based clustering.

with its outgroup *B. kipukae* (ARSEF7032) (100 genes). We found that the Clades A–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 measurements of the length and the width of phialides and conidia are reported per strain in Table S3. The average length and width of phialides and conidia of *B. asiatica* s.lat., *B. bassiana* s.lat. and all clades inside each of them can be found in Table S4. The length of phialides and conidia were significantly different between *B. asiatica* and *B. bassiana* (one-factor ANOVA; phialide length: F = 16.858, p-value = $4.324e^{-04}$; conidia length: F = 79.566, p-value = $6.309e^{-09}$) but the other traits were not significantly different between the two species (phialide width: F = 0.199, p-value = 0.659; conidia width: F = 79.566, p-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-value = 0.02, Fig. 3b). For *B. bassiana*, none of the examined traits was significantly different between clades (Table S4) (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* and *B. vermiconia* were found very close to *B. asiatica* (Fig. 4b, top panel). The intraspecific clades were not well separated based on these methods.

The distribution pattern of secondary metabolite production corresponded broadly to the presence/absence pattern of secondary metabolite gene clusters (SMGCs) among the species (Fig. 5). There were 41 SMGCs inferred from the reference genome (Table S4, S5), of which 15 were conserved among all the isolates. Twenty-five *Beauveria* isolates possess all the SMGCs most of which belong to *B. bassiana* s.lat. The three clades within *B. bassiana* s.lat. did not show distinct clustering patterns with most of the strains maintaining all SMGCs, except Cluster 37 (Indole-Nrps) that was randomly lost among some strains (Fig. 5). The isolates of *B. asiatica* s.lat. grouped together with a few SMGCs deletions shared between them – Cluster1 (Terpene), Cluster11 (NRPS), Cluster41 (Terpene-NRPS). No distinction could be observed between the two clades of *B. asiatica*. The other species included tended to group together outside the two species complexes but some of them were scattered among *B. asiatica* (Fig. 5).

Taxonomy

Based on genomic and morphological 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 838940; Fig. 6

Etymology. Name derived from the location where the type specimen was found, Nam Nao National Park, Nam Nao District.

Holotypus. Thailand, Phetchabun Province, Nam Nao District, Nam Nao National Park, Headquarter Nature Trail, N16.74° E101.57°, on adult of *Xylocopa latipes* (*Hymenoptera*, *Apidae*), 4 July 2012, *A. Khonsanit*, *K. Tasanathai*, *P. Srikitikulchai*, *S. Mongkolsamrit & W. Noisripoom* (holotype BBH 36158; ex-holotype strain BCC 64218).

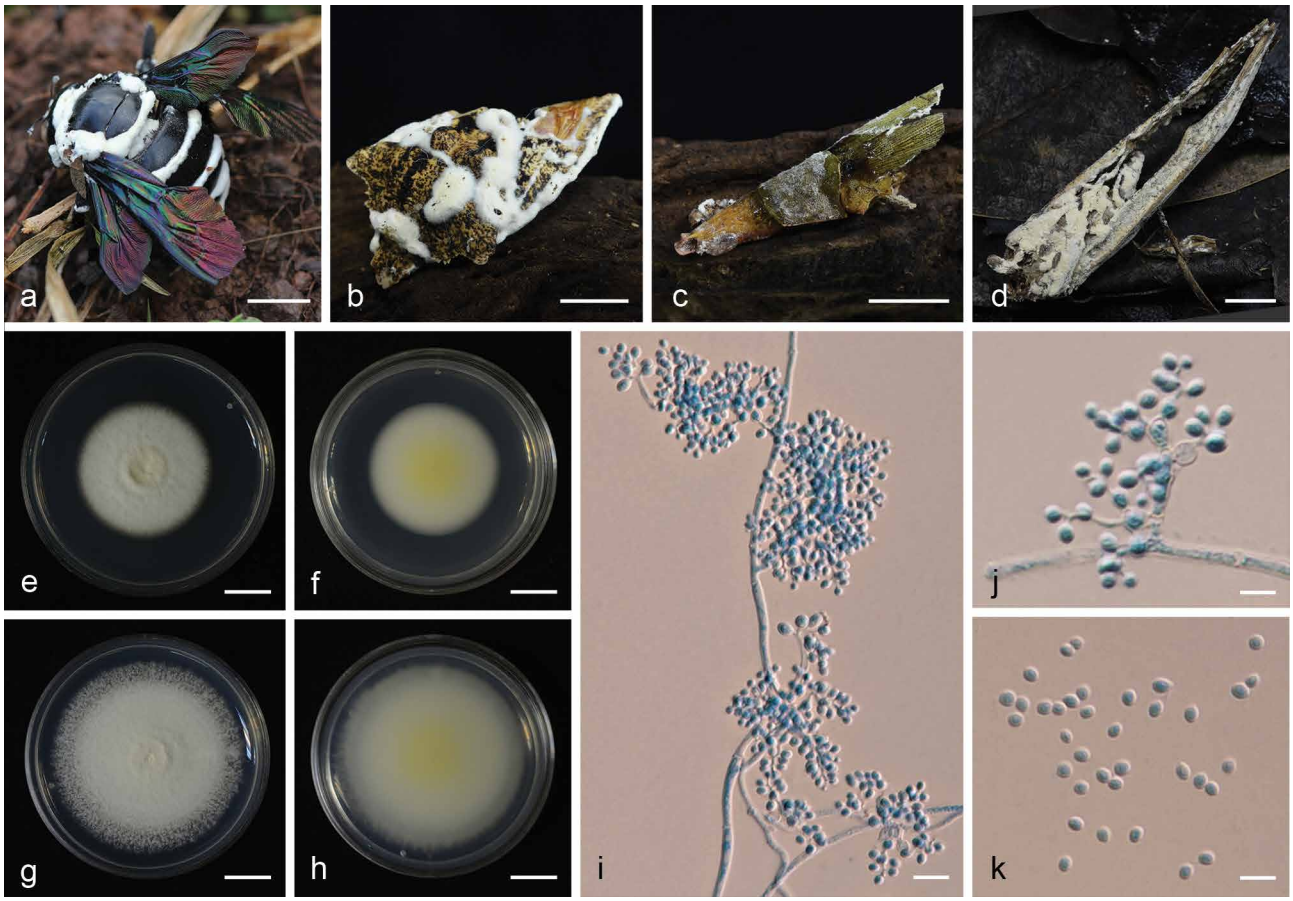

Fig. 6 *Beauveria namnaoensis*. a–d. Fungus on the hosts; e. colony obverse on PDA after 10 d; f. colony reverse on PDA after 10 d; g. colony obverse on PDA after 20 d; h. colony reverse on PDA after 20 d; i–j. phialides and conidia; k. conidia. — Scale bars: a, e–h = 10 mm; b–d = 5 mm; i = 10 µm; j–k = 5 µm.

Sexual morph — Unknown.

Asexual morph — Hosts covered with white mycelium, powdery when sporulating. *Phialides* hyaline, solitary, smooth-walled, base ampulliform, clavate, globose, mucronate, pyriform, subglobose, subspherical to lageniform (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 — Colony growing at room temperature attaining 29–31 mm diam 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.

Hosts — Specimens found on blue milkweed beetle (*Coleoptera*), stink bug (*Hemiptera*, *Pentatomidae*), wasp (*Hymenoptera*), pupa and adult of moth (*Lepidoptera*) and grasshopper (*Orthoptera*).

Additional specimens examined. Thailand, Chanthaburi Province, Khao Soi Dao District, Khao Soi Dao Wildlife Sanctuary, N13.10° E102.19°, on stink bug (*Hemiptera*, *Pentatomidae*), 1 Jan. 2000, *B. Thongnuch*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai*, *S. Mongkolsamrit* & *W. Chaygate* (BBH 14343: BCC 18114); Kanchanaburi Province, Thong Pha Phum District, Thung Yai Naresuan Wildlife Sanctuary, Krathon Ruesi Nature Trail, N15.33° E98.92°, on moth adult (*Lepidoptera*), 2 Dec. 2005, *B. Thongnuch*, *K. Tasanathai*, *P. Srikitikulchai* & *W. Chaygate* (BBH 15191: BCC 19745); Phetchaburi Province, Kaeng Krachan District, Kaeng Krachan National Park, Ban Krang Camp Nature Trail, N12.88° E99.63°, on blue milkweed beetle (*Coleoptera*), 26 Apr. 2006, *B. Thongnuch*, *K. Tasanathai*, *R. Ridkaew* & *W. Chaygate* (BBH 18375: BCC 21293); Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Bueng Phai Nature Trail, N14.44° E101.37°, on wasp (*Hymenoptera*), 29 Nov. 2006, *B. Thongnuch*, *P. Puyngain*, *T. Keokene* & *W. Chaygate* (BBH 23082: BCC 23823); Chiang Mai Province, Doi Inthanon National Park, Mae Chaem District, Mae Chaem Junction (KM.38) Nature Trail, N18.54° E98.52°, on grasshopper (*Orthoptera*), 27 Nov. 2008, *A. Khonsanit*, *K. Tasanathai* & *P. Srikitikulchai* (BBH 25297: BCC 34350).

Notes — *Beauveria namnaoensis* is hardly distinguishable morphologically from other closely related species such as

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

Species	Host/substrate	Phialides (µm)	Conidia (µm)	Distribution	References
B. bassiana s.str.	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, USA	Rehner et al. (2011), Khonsanit et al. (2020), This study
B. namnaoensis 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
B. neobassiana 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

B. bassiana and *B. neobassiana* (Table 2 and refer to the results on morphological variation). However, this new species is supported as distinct based on phylogenetic analyses.

Beauveria neobassiana Khons., Kobmoo & Luangsa-ard, *sp. nov.* — MycoBank MB 838939; Fig. 7

Etymology. Name 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, N14.44° E101.37°, on adult cicada (*Hemiptera*), 20 May 1996, *K. Tasanathai*, *N.L. Hywel-Jones*, *S. Sivichai* & *S. Thienhirun* (holotype BBH 5054; ex-holotype strain BCC 1848).

Sexual morph — Unknown.

Asexual morph — Hosts covered with white mycelium, powdery while sporulating. *Phialides* hyaline, solitary, smooth-walled, base ovoid, subspherical (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 — Colony 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 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.

Hosts — Specimens found on adult beetles, weevils (*Coleoptera*), adult cicadas (*Hemiptera*), ants (*Hymenoptera*) and larvae and adult of moth (*Lepidoptera*).

Additional specimens examined. Thailand, Kanchanaburi Province, Sangkhla Buri District, Khao Laem National Park, Sum Nuk Buhd To, N15.02° E98.60°, on adult beetle (*Coleoptera*), 21 June 1995, *N.L. Hywel-Jones*, *R. Nasit* & *S. Sivichai* (BBH 4614: BCC 1446); Phetchaburi Province, Kaeng Krachan District, Kaeng Krachan National Park, Khlong 1 Nature Trail, N12.88° E99.63°, on moth (*Lepidoptera*), *J.J. Luangsa-ard*, *K. Tasanathai*, *N.L. Hywel-Jones*, *P. Srikitikulchai*, *R. Nasit*, *S. Mongkolsamrit* & *W. Chaygate* (BBH 14301); Chiang Mai Province, Mae Chaem District, Doi Inthanon National Park, N18.54° E98.52°; on ant (*Hymenoptera*), 1 Jan. 2000, *B. Thongnuch*, *K. Tasanathai*, *P. Srikitikulchai*, *R. Ridkaew*, *S. Mongkolsamrit* & *W. Chaygate* (BBH 14373: BCC 18124, BBH 14374: BCC 18171); Phetchabun Province, Nam Nao District, Nam Nao National Park, trail to nature study area, N16.74° E101.57°, on weevil (*Coleoptera*, *Curculionoidea*), 23 May 2000, *K. Tasanathai*, *P. Lutthisungneon*, *R. Nasit* & *S. Sivichai* (BBH 7666: BCC 2660); Tak Province, Umphang District, Umphang Wildlife Sanctuary, Pi Tu Kro Waterfall Nature Trail, N15.92° E98.76°, on queen ant (*Hymenoptera*), 26 June 2001, *A. Khonsanit*, *B. Thongnuch*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai*, *S. Mongkolsamrit* & *W. Chaygate* (BBH 23919: BCC 31619); Chiang Mai Province, Fang District, Doi Phahompok National Park, Doi Phahompok Nature Trail, N20.00° E99.14°, on queen ant (*Hymenoptera*), 5 Feb. 2006, *B. Thongnuch*, *K. Tasanathai*, *S. Mongkolsamrit* & *W. Chaygate* (BBH 16599: BCC 20197); Nakhon Ratchasima Province, Pak Chong District,

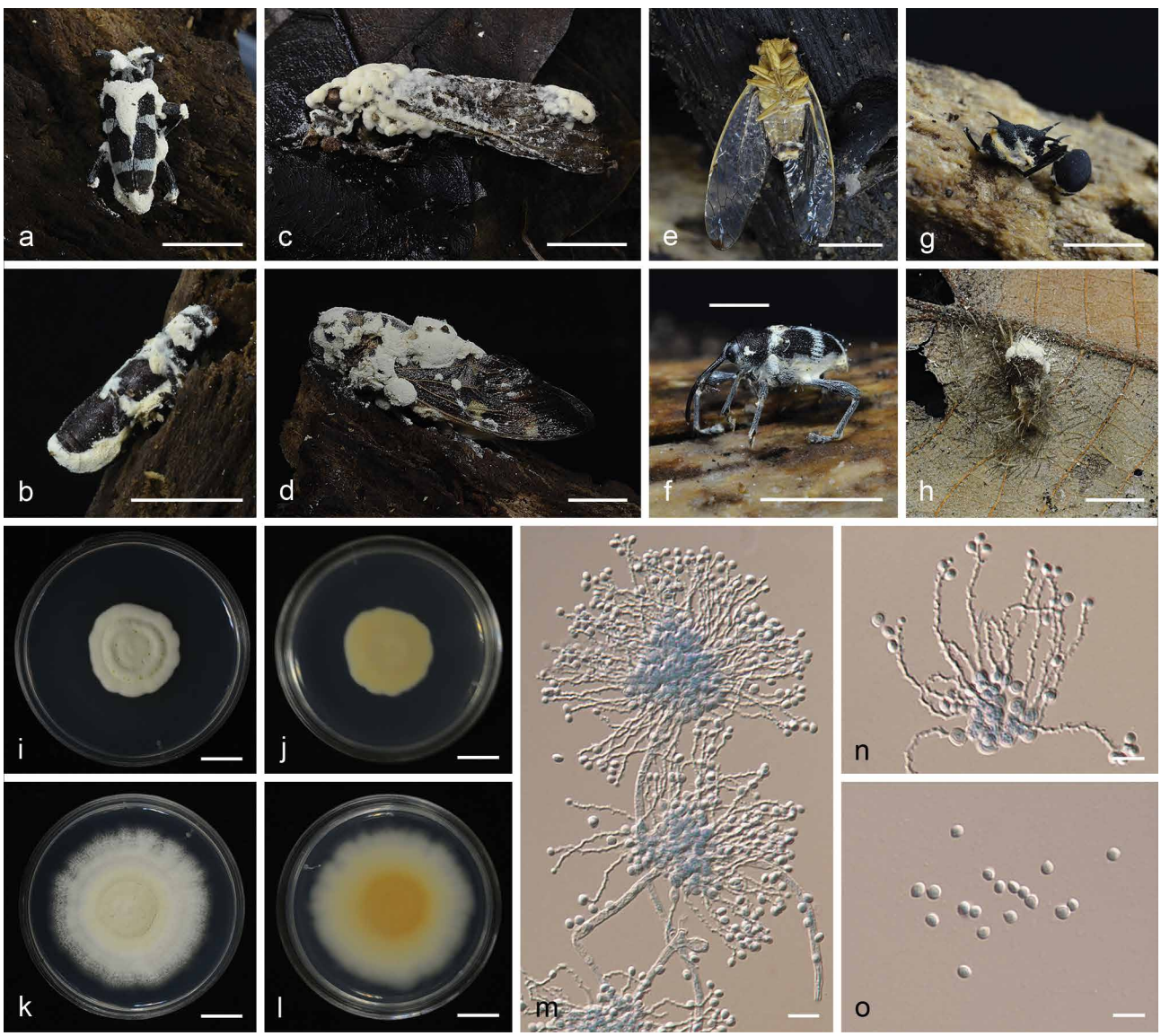

Fig. 7 *Beauveria neobassiana*. a–h. Fungus on the hosts; i. colony obverse on PDA after 10 d; j. colony reverse on PDA after 10 d; k. colony obverse on PDA after 20 d; l. colony reverse on PDA after 20 d; m–n. phialides and conidia; o. conidia. — Scale bars: a–b, d–h = 5 mm; c, i–l = 10 mm; m = 10 µm; n–o = 5 µm.

Khao Yai National Park, Kong Kaeo Waterfall Nature Trail, N14.44° E101.37°, on adult beetle (*Coleoptera*), 12 June 2007, *C. Chuaseeharonnachai*, *S. Sivichai* & *S. Mongkolsamrit* (BBH 24543: BCC 25950); Chanthaburi Province, Soi Dao District, Khao Soi Dao Wildlife Sanctuary, Headquarter Nature Trail, N13.10° E102.19°, on adult cicada (*Hemiptera*), 22 July 2008, *A. Khonsanit*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai* & *S. Mongkolsamrit* (BBH 24402: BCC 30545), on adult beetle (*Coleoptera*), 22 July 2008, *A. Khonsanit*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai* & *S. Mongkolsamrit* (BBH 23856: BCC 31604); Chiang Rai Province, Chiang Khong District, Phlu Kaeng Waterfall Nature Trail, N20.26° E100.39°, on *Lepidoptera* larva (*Lepidoptera*), 17 Jan. 2009, *A. Khonsanit*, *K. Tasanathai*, *P. Srikitikulchai* & *S. Mongkolsamrit* (BBH 26635: BCC 35935); Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Mo Sing To Nature Trail, N14.44° E101.37°, on adult beetle (*Coleoptera*), 20 July 2009, *K. Tasanathai*, *P. Srikitikulchai*, *R. Ridkaew*, *S. Mongkolsamrit* & *T. Chohmee* (BBH 26358: BCC37372); ibid., 16 Aug. 2009, *K. Tasanathai*, *P. Srikitikulchai*, *R. Ridkaew*, *S. Mongkolsamrit* & *T. Chohmee* (BBH 26797: BCC 37938); Chanthaburi Province, Pong Nam Ron District, Suchin's Orchard, N12.92° E102.39°, on adult beetle (*Coleoptera*), 16 Dec. 2014, *A. Khonsanit*, *D. Thanakitpipattana*, *N. Wiriyathanawudhiwong* & *W. Noisripoom* (BBH 39718: BCC 76579).

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

Beauveria thailandica Khons., Kobmoo & Luangsa-ard, *sp. nov.* — MycoBank MB 838941; Fig. 8

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

Holotype. Thailand, Chanthaburi Province, Soi Dao District, Khao Soi Dao Wildlife Sanctuary, N13.10° E102.19°, on *Coleoptera* larva (*Coleoptera*), 1 Jan. 2000, *N.L. Hywel-Jones* (holotype BBH 13831; ex-type culture BCC 16585; TBRC 8350).

Sexual morph — *Stromata* arising from the posterior part of *Coleoptera* larva, cylindrical with rounded apices, yellow (8B–8A) when fresh and yellow-orange (19B–19A) when dry, 38–50 × 0.8–2.5 mm. *Perithecia* semi-immersed, ovoid, (420–)469.6–539 (–580) × (150–)182.2–258.5(–290) µm (n = 30). *Asci* hyaline, cylindrical, capitate, (180–)215.4–299.5(–335) × (3–)3.2–4.7(–6) µm (n = 50). *Asci-caps* hyaline, hemispherical, (2–)2.2–3.1(–3) × (3.5–)3.6–4.1(–4) µm (n = 30). *Ascospores* hyaline, filiform, multiseptate, 241–320 × 1 µm (n = 8), breaking into 64 part-spores. *Part-spores* hyaline, cylindrical with truncated end, (4–)5.2–12.9(–23) × 1 µm (n = 50).

Asexual morph — Hosts covered with white mycelium, powdery when sporulating. *Phialides* hyaline, solitary, smooth-walled, base ampulliform, mucronate, rostrate, subspherical to lageniform (2–)2.5–3.7(–5) × (1.5–)2–3(–4) µm (n = 150). *Conidia* hyaline, smooth-walled, obovoid, ovoid, occasionally globose to subglobose (2.5–)3.1–4.3(–6) × (1.5–)1.6–2.3(–3) µm (n = 150).

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

Hosts — Specimens found on ladybugs, longhorn beetle (*Coleoptera*), earwigs (*Dermaptera*), adult cicada, leafhoppers, stink bug (*Hemiptera*), queen ant and wasps (*Hymenoptera*).

Additional specimens examined. Thailand, Phetchabun Province, Nam Nao District, Nam Nao National Park, Lum Nam Cheun Nature Trail, N16.74° E101.57°, on stink bug (*Hemiptera*, *Podopidae)*, 11 Oct. 1994. *N.L. Hywel-Jones*, *R. Nasit* & *S. Sivichai* (BBH 4362: BCC 1442); Phetchaburi Province, Kaeng Krachan District, Kaeng Krachan National Park, KM. 15 on road to Tor Tip Waterfall, N12.88° E99.63°, on adult cicada (*Hemiptera*), 23 May 1995, *N.L. Hywel-Jones* (BBH 4541: BCC 1654); ibid., on adult beetle (*Coleoptera*), 23 May 1995, *N.L. Hywel-Jones* (BBH 4542: BCC 1655); ibid., on earwigs (*Dermaptera*, *Chelisochidae*), 25 May 1995, *N.L. Hywel-Jones* (BBH 4563: BCC 2044); Kanchanaburi Province, Sangkhla Buri District, Khao Laem National Park, Sum Nuk Buhd To, N15.02° E98.60°, on adult beetle (*Coleoptera*), 21 June 1995, *N.L. Hywel-Jones*, *R. Nasit* & *S. Sivichai* (BBH4561: BCC1665); Chanthaburi Province, Khao Soi Dao District, Khao Soi Dao Wildlife Sanctuary, Behind office on the Nature Trail, N13.10° E102.19°, on *Lepidoptera* larva (*Lepidoptera*), 20 June 1996, *K. Tasanathai*, *R. Nasit* & *S. Sivichai* (BBH 5182: BCC 1906); Phetchabun Province, Nam Nao District, Nam Nao National Park, behind office to bungalow 1 Nature Trail, N16.74° E101.57°, on *Coleoptera* larva (*Coleoptera*), 20 Aug. 1996, *K. Tasanathai*, *R. Nasit* & *S. Sivichai* (BBH 5227: BCC 2086); Phetchaburi Province, Kaeng Krachan District, Kaeng Krachan National Park, KM. 15 on road to Tor Tip Waterfall, N12.88° E99.63°, on weevil bug (*Coleoptera*, *Curculionidae*), 2 June 2000, *N.L. Hywel-Jones* (BBH 7769: BCC 2676); Mae Hong Son Province, Mae Hong Son Road marker KM. 5.7, N19.30° E97.97°, on earwigs (*Dermaptera*, *Chelisochidae*), 30 June 2002, *N.L. Hywel-Jones & R. Nasit*, (BBH 16766: BCC 12907); Surat Thani Province, Phanom District, Khao Sok National Park, Sip Et Shin Waterfall Nature Trail, N8.99° E98.63°, on adult beetle (*Coleoptera*), 30 Sept. 2003, *K. Tasanathai*, *N.L. Hywel-Jones* & *S. Sivichai* (BBH 5353: BCC 2120); Phetchabun Province, Nam Nao District, Nam Nao National Park, Headquarter Nature Trail, N16.74° E101.57°, on adult leafhoppers (*Hemiptera, Cicadellidae*), 30 June 2004, *B. Thongnuch*, *K. Tasanathai* & *W. Chaygate* (BBH 10140: BCC 16183); Tak Province, Umphang District, Umphang Wildlife Sanctuary, Pi Tu Kro Waterfall (Preto Lo Su), N15.92° E98.76°, on adult longhorn beetle (*Coleoptera*, *Cerambycidae*), 26 June 2008, *A. Khonsanit*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai* & *S. Mongkolsamrit* (BBH 23918: BCC 31618); Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Mo Sing To Nature Trail, N14.7116666666667 E101.421666666667, sexual morph emerging between head and thorax of adult click beetle (*Coleoptera*, *Elateridae*), 18 June 2009, *N.L. Hywel-Jones*, *K. Tasanathai*, *R. Ridkaew*, *S. Mongkolsamrit* & *T. Chohmee* (BBH 38825: BCC 36657); Chanthaburi Province, Khao Soi Dao District, Khao Soi DaoWildlife Sanctuary, N13.10° E102.19°, on longhorn beetle (*Coleoptera*), 21 Aug. 2009, *B. Thongnuch*, *J.J. Luangsa-ard*, *K. Tasanathai*, *P. Srikitikulchai*, *S. Mongkolsamrit* & *W. Chaygate* (BBH 14344: BCC 18115); Nakhon Ratchasima Province, Pak Chong District, Khao Yai National Park, Mo Sing To Nature Trail, N14.44° E101.37°, on *Coleoptera* larva (*Coleoptera*), 27 Sept. 2011, *A. Khonsanit*, *K. Sansatchanon*, *K. Tasanathai*, *S. Mongkolsamrit* & *W. Noisripoom* (BBH 32175: BCC 49762); Chiang Mai Province, Chiang Dao District, Chiang Dao Wildlife Sanctuary, Chiang Dao Wildlife Research Station, N19.39° E98.84°, on *Lepidoptera* larva (*Lepidoptera*), 5 Oct. 2012, *A. Khonsanit*, *K. Tasanathai*, *P. Srikitikulchai*, *R. Promharn* & *W. Noisripoom* (BBH 38847: BCC 56283); Chiang Mai Province, Chiang Dao District, Ban Hua Thung Community Forest, N19.39° E98.84°, on adult wasp (*Hymenoptera*), 31 Oct. 2014, *A. Khonsanit*, *D. Thanakitpipattana*, *K. Tasanathai*, *P. Srikitikulchai*, *S. Wongkanoun* & *W. Noisripoom* (BBH 40622: BCC 76509).

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

DISCUSSION

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

Phylogenetics species criterion

The intraspecific genetic groups as found in these two species complexes by Khonsanit et al. (2020) were recovered by population genomics data in this study. The main question is whether they should be considered as distinct species. The evolutionary species concept (ESC) defines a species as 'a single lineage of ancestor-descendant populations which maintain its identity from other such lineages, and which has its own evolutionary

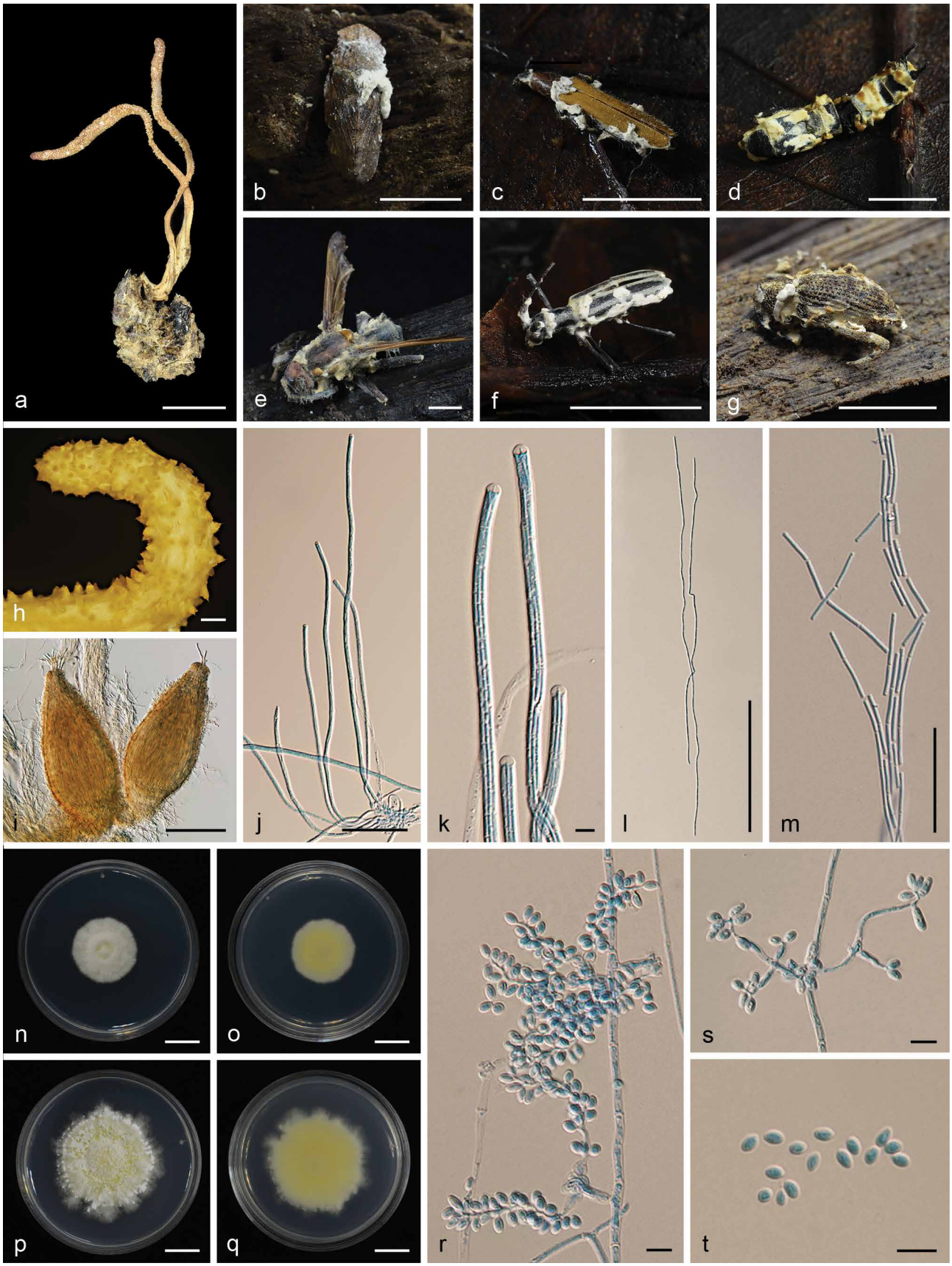

Fig. 8 *Beauveria thailandica*. a. Stromata on host (*Coleoptera* larva); b–g. fungus on the hosts; h. fertile head; i. perithecia; j. asci; k. asci with asci caps; l. ascospore; m. part-spores; n. colony obverse on PDA in 10 d; o. colony reverse on PDA in 10 d; p. colony obverse on PDA in 20 d; q. colony reverse on PDA in 20 d; r–s. phialides and conidia; t. conidia. — Scale bars: a, n–q = 10 mm; b–g = 5 mm; h = 1 mm; i = 200 µm; j, l = 50 µm; k, r–t = 5 µm; m = 10 µm.

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)	Phialides (µm)	Conidia (µm)	Distribution	References
B. asiatica s.str.	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	Rehner et al. (2011), Khonsanit et al. (2020)
B. thailandica 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

tendencies and historical fate' (Wiley 1978). This concept is not debated but is not an operational species delimitation criterion (De Queiroz 2007, Giraud et al. 2008). For fungi, the so-called phylogenetic species concept (PSC) is an operational concept that has been predominant and proposed as the most consistent to ESC (Taylor et al. 2000). According to the theoretical concept related to the most common ancestor and descending progenies, PSC is naturally associated to the monophyly and consistency of clades across genes, which became the basis to phylogenetic species recognition (PSR) (Cracraft 1983, Mishler & Brandon 1987, Mishler & Theriot 2000). According to this criterion, *B. asiatica* Clade C, Clade Mixed and *B. bassiana* Clade A–C should be considered as distinct species. However, monophyly-based species recognition received also some criticisms; monophyly-based species recognition cannot apply to asexual species because, without recombination, all lineages are monophyletic and with consistent gene genealogies. *Beauveria* had actually long been thought to be an asexual genus (Basalmo-Crivelli 1835). Our analyses of linkage disequilibrium (LD) decay suggested substantial recombination within some of these clades, which is consistent to observations of sexual morphs for several *Beauveria* species, including *B. bassiana* (Rehner et al. 2011, Khonsanit et al. 2020). The phylogenetic networks which take into account reticulated evolution still showed clear separation between them.

However, PSC can be arbitrary in regard of the limit of where to put species boundaries as any kind of molecular markers with polymorphisms would allow grouping individuals into monophyletic clades based on their respective alleles. PSC was thus proposed to rely on the concordance between distinct gene genealogies (Avis & Ball 1990, Baum & Shaw 1995), resulting in the genealogical concordance phylogenetic species recognition (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 actually had 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 chemotaxonomy and morphology

Chemotaxonomy is an approach of classifying and identifying microorganisms based upon the similarities and differences in biochemical compositions. In filamentous fungi, chemotaxonomy in the broadest sense usually involves secondary metabolites which have been extensively studied particularly in *Ascomycota*. *Penicillium* was the first asexual genus to be chemotaxonomically examined (Frisvad 1981). Therein, thin-layer chromatographic profiles of mycotoxins in combination with classical taxonomy allowed the recognition of four new groups within *Penicillium* subg. *Penicillium*. Later, several genera including *Alternaria* (Andersen et al. 2008), *Aspergillus* (Frisvad & Samson 1990, Samson et al. 2004, Frisvad & Larsen 2015), *Talaromyces* (Frisvad et al. 1990), *Fusarium* (Thrane & Hansen 1995, Schmidt et al. 2004, Zain 2010), *Stachybotrys* (Andersen et al. 2003), *Trichoderma* (Thrane et al. 2001) and many more, were subjected to chemotaxonomic examinations showing highly species-specific metabolic profiles. The chemotaxonomic concept has also been proven to be successfully applied in polyphasic taxonomy within xylarialean fungi which led to the discovery of potential chemotaxonomic markers (Stadler

et al. 2001a, b, 2003, 2014, Stadler & Hellwig 2005, Kuhnert et al. 2017, Kuephadungphan et al. 2021). For instance, sporothric acid, isosporothric acid and dihydroisosporothric acid appeared to be specific to *Hypoxylon monticulosum* (Surup et al. 2014), viridistratin A–C to *Annulohypoxylon viridistratum* (Becker et al. 2020), Minutellins A–D to *Annulohypoxylon minutellum* (Kuhnert et al. 2017) and Ienormandin A–G to *Hypoxylon jaklitschii* and *Hypoxylon lenormandii* (Kuhnert et al. 2015).

Considering *Beauveria*, chemotaxonomy was adopted and applied more than three decades ago (Mugnai et al. 1989, Bridge et al. 1990), revealing chemical profiles corresponding more or less to species based on morphological criteria, but were heterogeneous and overlapping between populations within species (e.g., *B. bassiana*). Recently, Berestetskiy et al. (2018) demonstrated that the chromatographic profiles could be used to distinguish *B. bassiana* from *B. pseudobassiana* while Yin et al. (2020) expressed doubts on using chemotaxonomy for species identification in *Beauveria*. The latter study showed that, although the two species appeared to produce structurally different congeners of the cyclodepsipeptide beauveriolides, they also produced similar compounds found in fungi from other genera. This is not surprising since several fungal species, not only *Beauveria*, can have abundant compounds in common. 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. This pattern reflected a conservation of all secondary metabolite gene clusters shared between most of the *B. bassiana* s.lat. strains while some were lost in *B. asiatica* s.lat. and other species. However, between the clades within each species complex, no distinctive discriminatory pattern was found.

It is still too early to conclude that the chemotaxonomic concept has failed to discriminate these clades apart as the secondary metabolites were only obtained under a single growth condition. The YMG medium used in our study has been proven to allow expression of many species-specific metabolites in various ascomycetes and is even used to reveal the existence of cryptic species in certain genera, i.e., *Xylaria* (Stadler et al. 2003, Kuephadungphan et al. 2021). The metabolic profiles derived from YMG seemed to allow discrimination only between some *Beauveria* species in our study. As culture media composition has influences on secondary metabolite production, other media should be evaluated. For now, it can thus be concluded that the metabolic profiles generated under these conditions could only be used to distinguish *B. asiatica* from other *Beauveria* species with some exceptions.

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 of *Beauveria* (Rehner & Buckley 2005, Imoulan et al. 2017, Khonsanit et al. 2020). Our study revealed that the morphological differences among clades within *B. asiatica* and *B. bassiana* were much less evident than the genomic data. Nevertheless, the morphology, particularly the conidial length, was significantly different between *B. asiatica* Clade C and Clade Mixed (conidial length). Altogether, our findings support the idea that the two *Beauveria* species are composed of sibling species with similar phenotypes, i.e., cryptic species.

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 ex-type strain ARSEF1564 can be considered as *B. bassiana* s.str. Regarding *B. asiatica* s.lat., the Clade Mixed is proposed here as a new species, *B. thailandica*, and the Clade C containing the ex-type strain ARSEF4850 remains *B. asiatica* s.str. 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 studies

Investigating the sexual compatibility between closely related species within each of the two species complexes should be conducted in 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* spp: Lombard et al. 2010, Li et al. 2020) or sexually compatible with post-mating isolation (e.g., *Microbotryum* spp.: De Vienne et al. 2009). *Beauveria* was initially proposed to be an asexual 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.

CONCLUSIONS

Our work showed that whole-genome sequence 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. 2020a, *Ophiocordyceps*: Kobmoo et al. 2012, 2019, Araújo et al. 2018, Khonsanit et al. 2019). Many taxonomic studies 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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Supplementary material

Fig. S1 Analyses of recombination footprint for: a. *Beauveria asiatica*; b. *B. bassiana*. 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* s.lat. (left panel) and *B. bassiana* s.lat. (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.

Table S2 Fermentation periods for the production of secondary metabolites of *Beauveria* strains used to study chemotaxonomy.

Table S3 Average width and length (µm) of conidia and phialides per strain for the strains used in statistical analyses of morphological traits.

Table S4 The results of statistical analyses on morphological traits. a. The average length and width of phialides and conidia among *Beauveria* species; b. ANOVA tests of difference between clades within *B. asiatica* and *B. bassiana*.

Table S5 Characteristics of secondary metabolite gene clusters inferred on the genome reference of *Beauveria bassiana* ARSEF8028.