Taxonomic and phylogenetic characterizations reveal four new species of *Simplicillium* (Cordycipitaceae, Hypocreales) from Guizhou, China

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*Simplicillium* species are commonly found from soil, seawater, rock surface, decayed wood, air and as symbiotic, endophytic, entomopathogenic and mycoparasitic fungi. Minority insect-associated species was reported. *Simplicillium coccinellidae*, *S. hymenopterorum*, *S. neolepidopterorum* and *S. scarabaeoidea* were introduced as the newly insect-associated species. The phylogenetic analyses of two combined datasets (LSU + RPB1 + TEF and SSU + ITS + LSU) revealed that *S. coccinellidae* and *S. hymenopterorum* were both nested in an independent clade. *S. neolepidopterorum* and *S. scarabaeoidea* have a close relationship with *S. formicidae* and *S. lepodopterorum*, respectively. *S. neolepidopterorum* can be easily distinguished from *S. formicidae* by ellipsoidal to cylindrical, solitary conidia which occasionally gather in short imbricate chains. *S. scarabaeoidea* could be easily distinguished from *S. lepodopterorum* by having longer phialides and larger conidia. Based on the morphological and phylogenetic conclusion, we determine the four newly generated isolates as new species of *Simplicillium* and a new combination is proposed in the genus *Leptobacillium*.

The genus *Simplicillium* was established for the typical species *S. lanosoniveum* (J.F.H. Beyma) Zare & W. Gams and three other species *S. obclavatum* (W. Gams) Zare & W. Gams, *S. lamellicola* (F.E.V. Sm.) Zare & W. Gams and *S. wallacei* H.C. Evans1. The typical characteristic of *Simplicillium* is its solitary phialides, which could be easily distinguished from its closely genus *Lecanicillium* W. Gams & Zare. *S. wallacei* was transferred to the genus *Lecanicillium* based on the phylogenetic analysis by Zare & Gams2. Fourteen species were reported later. Okane et al.3 transferred *S. chinense* F. Liu & L. Cai and *S. coffeaeum* A.A.M. Gomes & O.L. Pereira to the genus *Leptobacillium* and this transfer was confirmed by Wang et al.4. *Simplicillium* species have diverse ecology, but most species are known from few strains impeding to define their habitat and ecology accurately. Species were found from soil (e.g., *S. cylindrosporum*, *S. minutensis*, *S. subtropicum*, and *S. symphyophorum*), as plant endophyte (e.g. *S. coffeaum* and *S. filiforme* isolated from Coffea arabica and *Citralis lanatus*), from decaying wood or rock (*S. calcicola* and *S. chinense*), or from multiple sources. *Simplicillium obclavatum* was isolated from air, soil, bark, human nail, and seawater1,10, whereas *S. aogashimaense* was isolated from soil, seawater, and as symbiotic fungi from Nilaparvata lugens Stål11,12. *Simplicillium lamellicola* was isolated as endophytic, entomopathogenic, and mycoparasitic fungi13–15. *Simplicillium lanosoniveum* was isolated as cyanobacterium-symbiotic, endophytic, entomopathogenic, and mycoparasitic fungi16–19. Among those *Simplicillium* species, six species viz. *S. cicadellidae*, *S. formicae*, *S. formicidae*, *S. lamellicola*, *S. lanosoniveum* and *S. lepidopterorum*, were found associated with insects.

In the present study, four novel insect-associated species viz. *Simplicillium coccinellidae*, *S. hymenopterorum*, *S. neolepidopterorum* and *S. scarabaeoidea*, were introduced based on morphological comparison and molecular phylogenetic analyses, and this may contribute to the control of insect pest and the discovery of useful novel compounds.

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Result

Phylogenetic analyses. In the phylogenetic tree, *Purpureocillium lilacinum* (Thom) Luangsard, Houbraken, Hywel-Jones & Samson (CBS 284.36 and CBS 431.87) and *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (CBS 103.63) were used as the outgroup in analysis 1 and analysis 2, respectively. The concatenated sequences of analysis 1 and analysis 2 included 46 and 22 taxa, and consisted of 1,729 (LSU: 497, RPB1: 550 and TEF: 682) and 1,904 (SSU: 845, ITS: 541 and LSU: 518) characters with gaps, respectively.

Analysis 1: The P-value of PAUP4.0b10 using the command “hompart” is 0.01, and indicated the dataset SSU + RPB1 + TEF is not suitable for the combined analysis. The selected model for LSU, RPB1 and TEF were SYM + G4, SYM + G4 and GTR + F + I + G4, respectively. The final value of the highest scoring tree was –17,856.725706, which was obtained from the ML analysis of the dataset (LSU + RPB1 + TEF). The parameters of GTR model to analysis of the dataset were estimated base frequencies; $A_{0} = 0.235757, C_{0} = 0.286704, G_{0} = 0.270379$, $T = 0.207160$; substitution rates $AC = 0.874437, AG = 2.344268, AT = 0.877112, CG = 0.872563, CT = 6.144163$, $GT = 1.000000$; gamma distribution shape parameter $\alpha = 0.441982$. In the phylogenetic tree (Fig. 1), both analyses of ML and BI trees were largely congruent, and strongly supported in most branches. All *Simplicillium* species were nested in an independent clade, which was the earliest diverging lineage in Cordycipitaceae. The four new species, *S. coccinelldae*, *S. hymenopterorum*, *S. neolepidopterorum* and *S. scarabaeoidae* were both formed an independent branch and clustered with *S. cicadellidae*, *S. formicidae* and *S. lepidopterorum* in a subclade.

Analysis 2: The P-value of PAUP4.0b10 using the command “hompart” is 0.99, and indicated the dataset SSU + ITS + LSU is suitable for the combined analysis. The selected model was JC for SSU and K2P + G4 for ITS + LSU. The final value of the highest scoring tree was –6,637.139922, which was obtained from the ML analysis of the dataset (SSU + ITS + LSU). The parameters of GTR model to analysis of the dataset were estimated base frequencies; $A_{0} = 0.251177, C_{0} = 0.239762, G_{0} = 0.263036, T = 0.246025$; substitution rates $AC = 1.301732, AG = 2.440073, AT = 0.844382, CG = 1.306407, CT = 3.262235, GT = 1.000000$; gamma distribution shape parameter $\alpha = 0.552466$. In the phylogenetic tree (Fig. 2), both analyses of ML and BI trees were largely congruent, and strongly supported in most branches. Four well-supported clades representing four new novel species *S. coccinelldae*, *S. hymenopterorum*, *S. neolepidopterorum* and *S. scarabaeoidae* were obtained. These new species clustered with *S. cicadellidae*, *S. formicidae* and *S. lepidopterorum* in a well-supported subclade within the *Simplicillium* lineage. *S. coccinelldae* and *S. hymenopterorum* were both nested in an independent clade. *S. neolepidopterorum* and *S. scarabaeoidae* have a close relationship with *S. formicidae* and *S. lepidopterorum*, respectively.

Taxonomy

*Simplicillium coccinelldae* W.H. Chen, Y.F. Han, Z.Q. Liang sp. nov. (Fig. 3).

*Mycobank*: MB 835583.

*Etymology*: referring to its insect host, family Coccinellidae.

*Description*: The colonies were moderate-growing on PDA medium, reaching a diameter of 31–36 mm, in 14 days at 25 °C, convex, with white velutinate aerial mycelium, reverse yellowish to pale brown, especially in the middle, margin entire, soluble pigment not produced. *Vegetative hyphae* branched, hyaline, smooth-walled, septate, 1.1–1.9 μm wide. *Phialides* produced on aerial hyphae, always solitary, asceptate, hyaline, smooth-walled, relatively slender, and tapering toward the tip, 24.9–62.1 × 1.0–1.5 μm. *Conidia* in small subglobose slimy heads at the apex of the phialides, hyaline, cylindrical to globose, asceptate, smooth-walled, 1-celled, 2.0–3.4 × 1.6–2.0 μm, Octahedral crystals absent.

*Material examined*: CHINA, Guizhou, Guiyang, Duyun City (26°21′27″ N, 107°22′48″ E). On dead scarab (Coccinellidae), 1 October 2019, Wanhao Chen, DY10179 (GZAC DY10179, holotype), was deposited at the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China; ex-type living cultures, DY101791, DY101792. Sequences at the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China; ex-type living cultures, DY101791, DY101792. Sequences from isolated strain DY101791 has been deposited in GenBank with accession numbers: ITS = MT453861, LSU = MT453862 and TEF = MT471341.

*Know distribution*: China, Guizhou Province, Duyun City (26°21′27″ N, 107°22′48″ E).

*Notes*: *S. coccinelldae* share some similar conidial and phialide morphologies with the related species (Table 1). However, the pairwise dissimilarities of ITS sequences show 30, 127, 31, 29, 45, 33 bp difference within 584 bp between *S. coccinelldae* and *S. cicadellidae*, *S. formicidae*, *S. lepidopterorum*, *S. hymenopterorum*, *S. lepidopterorum*, *S. scarabaeoidae* respectively. Jeewan & Hyde recommended that a minimum of >1.5% nucleotide differences in the ITS regions may be indicative of a new species. Besides, based on the analysis of the combined dataset LSU + RPB1 + TEF and SSU + ITS + LSU, *S. coccinelldae* was nested in a separate group in both phylogenetic trees. Thus, the molecular phylogenetic results supported that *S. coccinelldae* was a new species in the genus *Simplicillium*.

*Simplicillium hymenopterorum* W.H. Chen, Y.F. Han, Z.Q. Liang sp. nov. (Fig. 4).

*Mycobank*: MB 835581.

*Etymology*: referring to its insect host, family Hymenoptera.

*Description*: The colonies were rapid-growing on PDA medium, reaching a diameter of 40–42 mm, in 14 days at 25 °C, convex, with white velutinate aerial mycelium, reverse pale yellow, especially in the middle, margin entire, soluble pigment not produced. *Phialides* produced on prostrate aerial hyphae, mainly solitary, asceptate, hyaline, smooth-walled, relatively slender, and tapering toward the tip, 19.3–46.2 × 1.1–2.3 μm. *Conidia* in small subglobose slimy heads at the apex of the phialides, hyaline, cylindrical to subellipsoidal, asceptate, smooth-walled, 1-celled, 2.1–2.8 × 1.3–1.9 μm, Octahedral crystals absent.

*Material examined*: CHINA, Guizhou, Guiyang, Duyun City (26°21′27″ N, 107°22′48″ E). On dead ant (Hymenoptera), 1 October 2019, Wanhao Chen, DY10169 (GZAC DY10169, holotype), was deposited at the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code,
GZAC), Guiyang City, Guizhou, China; ex-type living cultures, DY101691, DY101692. Sequences from isolated strain DY101691 has been deposited in GenBank with accession numbers: ITS = MT453848, SSU = MT453849, LSU = MT453850, RPB1 = MT471344 and TEF = MT471337.

Notes: Based on the analysis of the combined dataset LSU + RPB1 + TEF and SSU + ITS + LSU, *S. hymenopterorum* was nested in a separate group in two phylogenetic trees. The pairwise dissimilarities of ITS sequences show 105, 24, 31, 17 bp difference within 582 bp between *S. hymenopterorum* and *S. formicidae*, *S. lepodopterorum*, *S. lepidopterorum* and *S. hymenopterorum*.

![Figure 1. Phylogenetic relationships among the genus *Simplicillium* and closely-related species in Cordycipitaceae based on multigene dataset (LSU, RPB1 and TEF). Statistical support values (≥ 70%/0.7) are shown at the nodes for ML bootstrap support/BI posterior probabilities.](image-url)
coccinellidae, S. neolepidopterorum, respectively. The pairwise dissimilarities of RPB1 sequences show 25, 16 bp difference within 737 bp between S. hymenopterorum and S. cicadellidae, S. scarabaeoidea respectively. When compared with the typical characteristics of S. cicadellidae and S. scarabaeoidea (Table 1), S. hymenopterorum could be easily distinguished from S. cicadellidae and S. scarabaeoidea by having subglobose slimy heads of conidia, cylindrical to subellipsoidal conidia, 2.1–2.8 × 1.3–1.9 μm and phialides, 19.3–46.2 × 1.1–2.3 μm. Thus, morphologically based conclusion supported the molecular phylogenetic results that S. hymenopterorum was a new species in the genus Simplicillium.

Simplicillium neolepidopterorum W.H. Chen, Y.F. Han, Z.Q. Liang sp. nov. (Fig. 5).

MycoBank No.: MB 835582.

Etymology: referring to its insect host, order Lepidoptera.

Description: Insect host was completely covered by white to yellowish, loosely mycelium. Conidiophore mononematous. The colonies were slow-growing on PDA medium, reaching a diameter of 28–31 mm, in 14 days at 25 °C, convex, with white velutinate aerial mycelium, reverse yellowish to pale brown, especially in the middle, margin entire, soluble pigment not produced. Vegetative hyphae branched, hyaline, septate, smooth-walled, 1.3–1.4 μm wide. Phialides produced on aerial hyphae, always solitary and rather long and narrow, aseptate, hyaline, smooth-walled, relatively slender, and tapering toward the tip, 34.1–44.3 × 1.0–1.7 μm. Conidia solitary,
occasionally in short imbricate chains, hyaline, ellipsoidal to cylindrical, aseptate, smooth-walled, 1-celled, 2.5–3.8 × 1.5–2.1 μm, Octahedral crystals absent.

Material examined: CHINA, Guizhou, Guiyang, Duyun City (26°21′27.96″ N, 107°22′48.22″ E). On dead insect (Lepidoptera), 1 October 2019, Wanhao Chen, DY10175 (GZAC DY10175, holotype), was deposited at the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China; ex-type living cultures, DY101751, DY101752. Sequences from isolated strain DY101751 has been deposited in GenBank with accession numbers: ITS = MT453854, SSU = MT453856, LSU = MT453855 and TEF = MT471339.

Notes: Based on the analysis of the combined dataset SSU + ITS + LSU, S. neolepidopterorum is phylogenetically close to S. formicidae. Besides, the pairwise dissimilarities of ITS sequences show 153 bp difference within 580 bp between S. neolepidopterorum and S. formicidae. When compared with the typical characteristics of S. formicidae (Table 1), S. neolepidopterorum could easily distinguished from S. formicidae by having solitary conidia, occasionally in short imbricate chains, and ellipsoidal to cylindrical conidia. Thus, molecular phylogenetic results and morphologically based conclusion were supported S. neolepidopterorum was a new species in the genus Simplicillium.

Simplicillium scarabaeoidea W.H. Chen, Y.F. Han, Z.Q. Liang sp. nov. (Fig. 6).

MycoBank No.: MB 835580.

Etymology: referring to its insect host, family Scarabaeoidea.

Distribution: Insect host was completely covered by white, yellowish to pinkish, densely mycelium. Conidiophore monomorphous. The colonies were rapid-growing on PDA medium, reaching a diameter of 44–47 mm, in 14 days at 25 °C, convex, with white velutinate aerial mycelium; reverse pale yellow, margin entire, soluble pigment not produced. Phialides produced on prostrate aerial hyphae, mainly solitary, aseptate, hyaline, smooth-walled,
relatively slender, and tapering toward the tip, 18.5–63.4 × 1.1–1.4 μm. Conidia in small globose heads at the apex of the phialides, hyaline, ellipsoidal, aseptate, smooth-walled, 1-celled, 1.9–2.9 × 1.4–2.0 μm. Octahedral crystals absent.

Material examined: CHINA, Guizhou, Guiyang, Duyun City (26°21’27.96” N, 107°22’48.22” E). On dead insect (Lepidoptera), 1 October 2019, Wanhao Chen, DY10139 (GZAC DY10139, holotype), was deposited at the Institute of Fungus Resources, Guizhou University (formerly Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China; ex-type living cultures, DY101391, DY101392. Sequences from isolated strain DY101391 has been deposited in GenBank with accession numbers: ITS = MT453842, SSU = MT453843, LSU = MT453844, RPB1 = MT471343 and TEF = MT471335.

Notes: Based on the analysis of the combined dataset SSU + ITS + LSU, S. scarabaeoidea is phylogenetically close to S. lepodopterorum. However, the pairwise dissimilarities of RPB1 sequences show 31 bp difference within 760 bp between S. scarabaeoidea and S. lepodopterorum. When comparing with the typical characteristics of S. lepodopterorum (Table 1), S. scarabaeoidea could be easily distinguished from S. lepodopterorum by having longer phialides and larger conidia. Thus, molecular phylogenetic results and morphologically based conclusion were supported S. scarabaeoidea was a new species in the genus Simplicillium.

Figure 4. Simplicillium hymenopterorum (A) infected ant (Hymenoptera) (B,C) culture plate, showing the front (B) and the reverse (C) of the colony, cultured on PDA medium (D–I), (K) phialides solitary, conidia adhering ellipsoidal slimy head and conidia (J) conidia. Scale bars: 10 mm (B,C), 10 μm (D–K).

Figure 5. Simplicillium neolepidopterorum (A) infected moth (Lepidoptera) (B,C) culture plate, showing the front (B) and the reverse (C) of the colony, cultured on PDA medium (D–I) phialides solitary, conidia adhering ellipsoidal slimy head and conidia (J) conidia. Scale bars: 10 mm (B,C), 10 μm (D–J).
Leptobacillium filiform (R.M.F. Silva, R.J.V. Oliveira, Souza-Motta, J.L. Bezerra & G.A. Silva) W.H. Chen, Y.F. Han J.D. Liang & Z.Q. Liang, comb. nov.

Mycobank No.: MB839923.

Basionym: Simplicillium filiform R.M.F. Silva, R.J.V. Oliveira, Souza-Motta, J.L. Bezerra & G.A. Silva, Persoonia 41: 403 (2018).

Notes: Okane et al.3 transferred Simplicillium chinense and S. coffeanum to the genus Leptobacillium. In the present study, S. chinense, S. coffeanum and S. filiform were clustered into an independent clade (Fig. 2), and supported by Crous et al.7, Chen et al.20 and Wei et al.28. Thus, L. filiform is proposed as a new combination.

Discussion

Sung et al.40 refined the classification of Cordyceps and the Clavicipitaceae; the genus Simplicillium thus belongs to the Cordycipitaceae sensu stricto. The result of phylogenetic analysis of the combined dataset (SSU, LSU, RPB1, RPB2 and TEF) showed that Simplicillium species were all clustered in an independent group and as the most ancient lineage in the phylogenetic tree41. In this study, all Simplicillium species were also clustered into a clade at the end of the tree (Fig. 1) based on the analysis of the concentrated dataset (LSU, RPB1 and TEF). The four newly identified species, S. scarabaeoidea, S. hymenopterorum, S. neolepidopterorum and S. coccinellidae, were all clustered in a separate subclade. Liu & Cai36 reported a new species based on the morphological comparison and phylogenetic analysis of ITS and LSU sequences, which was the earliest application for the identification of Simplicillium species. Kondo et al.29 added the loci SSU in the analysis of Simplicillum species. Thus, three loci (ITS, LSU and SSU) were applied in the analysis of the relationship among Simplicillium species in this study.

The nutritional mode from plant to animals and fungi is the evolutionary characteristics of Hypocreales42. Plants associated fungi, which including living plants and plant residues were the common ancestor in the families Hypocreaceae and Clavicipitaceae41. The animal pathogenic fungi are likely inherited from the plant associated fungi by a series of interkingdom host jumps43. In the phylogenetic tree of analysis 2 (Fig. 2), S. chinense, S. filiforme and S. coffeanum were nested in a clade and at the end of the tree. The substrates of S. chinense, S. coffeanum and S. filiforme were decaying wood, branches of Coffea arabica and leaves of Citrullus lanatus44,45. All of them were belongs to plants associated fungi, and might reflect the initial state of Simplicillium species, which then underwent a host jump or transferred their nutritional preference. Simplicillium species have rich diversity.

Figure 6. Simplicillium scarabaeoidea (A) infected scarab (Scarabaeoidea) (B,C) culture plate, showing the front (B) and the reverse (C) of the colony, cultured on PDA medium (D–I) phialides solitary, conidia adhering ellipsoidal slimy head and conidia (J) conidia. Scale bars: 10 mm (B,C), 10 μm (D–J).
Numerous new secondary metabolites were found from *Simplicillium* species, such as alkaloids, diketopiperazines, and anthraquinones, especially aogacillin A, B and Simpotentin, which have antibacterial and antifungal activities and shown great potential applications in medicine. In addition, some *Simplicillium* species were isolated as symbiotic, entomopathogenic, and mycoparasitic fungi, and could be used to biocontrol of insect pest, nematode and microbial diseases. Thus, it is expected that useful novel compounds will be discovered from the newly-reported *Simplicillium* species described here and be a natural resource for the application in biocontrol, medicine and health.

**Materials and methods**

**Specimen collection and identification.** Four infected insect specimens (DY10139, DY10169, DY10175 and DY10179) were collected from Duyun City (26°21′24.71″ N, 107°22′48.22″ E), Guizhou Province, on 1 October, 2019. Isolation of strains was conducted as described by Chen et al. Fungal colonies emerging from specimens were isolated and cultured at 25 °C for 14 days under 12 h light/12 h dark conditions following protocols described by Zou et al. Accordingly, strains were obtained. The specimens and the isolated strains were deposited in the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China.

Macroscopic and microscopic morphological characteristics of the fungi were examined and the growth rates were determined from PDA cultures incubated at 25 °C for 14 days. Hyphae and conidiogenous structures were mounted in lactophenol cotton blue or 20% lactate solution and observed with an optical microscope (OM, DM4 B, Leica, Germany).

DNA extraction, polymerase chain reaction amplification and nucleotide sequencing.

DNA extraction was carried out by Fungal genomic DNA Extraction Kit (DP2033, BioTeke Corporation) in accordance with Liang et al. The extracted DNA was stored at −20 °C. The amplification of internal transcribed spacer (ITS) region, small subunit ribosomal RNA (SSU), large subunit ribosomal RNA (LSU) gene, RNA polymerase II largest subunit 1 (RPB1) and translation elongation factor 1 alpha (TEF) were amplified by PCR as described by White et al., Rakotonirainy et al., Castlebury et al. and van den Brink et al., respectively. PCR products were purified and sequenced at Sangon Biotech (Shanghai) Co. The generated sequences were submitted to GenBank.

**Sequence alignment and phylogenetic analyses.** Lasergene software (version 6.0, DNASTAR) was applied for the assembling and editing of DNA sequence in this study. The ITS, SSU, LSU, RPB1 and TEF sequences were downloaded from GenBank, based on Nonaka et al., Zhang et al., Gomes et al., Crous et al., Mongkolsamrit et al., Chen et al., Wei et al., Kondo et al. and others selected on the basis of BLAST algorithm-based searches in GenBank (Table 2). The Multiple datasets of ITS, LSU, SSU, RPB1 and TEF were aligned and edited by MAFFT v7.037b and MEGA6. Assembling of the combined datasets (LSU + RPB1 + TEF and SSU + ITS + LSU) were performed by SequenceMatrix v.1.7.8. The partition homogeneity test was conducted in PAUP4.0b10 by using the command “hompart”.

The datasets (LSU + RPB1 + TEF and SSU + ITS + LSU) were analysis by Bayesian inference (BI) and maximum likelihood (ML) methods and aimed to analysis of the relationship among *Simplicillium* species and its related species in the family Cordycipitaceae (analysis 1) and the relationship among *Simplicillium* spp. (analysis 2), respectively. For BI, a Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v.3.2.6 for the combined sequence datasets. The model for BI analysis was selected by ModelFinder in the software PhyloSuite. The Bayesian analysis resulted in 20,001 trees after 10,000,000 generations. The first 4000 trees, representing the burn-in phase of the analyses, were discarded, while the remaining 16,001 trees were used for calculating posterior probabilities in the majority rule consensus tree. After the analysis was finished, each run was examined using the program Tracer v1.5 to determine burn-in and confirm that both runs had converged. ML analyses were constructed with RAXMLGUI. The GTR+GAMMA model was used for all partitions, in accordance with recommendations in the RAXML manual against the use of invariant sites. The final alignment is available from TreeBASE under submission ID: 26290 (http://www.treebase.org).
| Species                      | Strain no. | GenBank accession no. |
|------------------------------|------------|-----------------------|
|                             |            | ITS          | SSU          | LSU          | RPB1         | TEF          |
| Akanthomyces aculeatus      | HUA 772    | KC519370     |              |              |              |              |
| A. attenuatus               | CBS 402.78 | AF339565     | EF468888     | EF468782     |              |              |
| A. coccidiopticercusclatus  | NHJ 6709   | EU369042     | EU369067     | EU369025     |              |              |
| A. farinosa                 | CBS 541.81 | IJQ425686    |              |              |              |              |
| A. kanyawimiae              | TBRC 7242  | MF140718     | MF140784     | MF140838     |              |              |
| A. kanyawimiae              | TBRC 7243  | MF140717     | MF140783     | MF140837     |              |              |
| A. kanyawimiae              | TBRC 7244  | MF140716     | MF140836     |              |              |              |
| A. lecanii                  | CBS 101247 | AF339555     | DQS22407     | DQS22359     |              |              |
| A. sulphureus               | TBRC 7247  | MF140720     | MF140841     |              |              |              |
| A. sulphureus               | TBRC 7248  | MF140722     | MF140787     | MF140843     |              |              |
| A. sulphureus               | TBRC 7249  | MF140721     | MF140786     | MF140842     |              |              |
| A. thailandicus             | TBRC 7245  | MF140719     | MF140840     |              |              |              |
| A. tuberculatus             | BCC 16819  | GQ249987     |              |              |              |              |
| A. tuberculatus             | OSC 6355   | AY886544     | DQS27324     | DQS27350     |              |              |
| Blackwellomyces cardinalis  | OSC 93609  | AY184962     | DQS223270    | DQS223325    |              |              |
| B. cardinalis               | OSC 93610  | AY184963     | EF469088     | EF469039     |              |              |
| B. pseudomilitaris          | NBRC 10140 | JN941393     | JN992482     |              |              |              |
| Cordyceps bifussispora      | EFCC 5690  | EF468806     | EF468854     | EF468746     |              |              |
| C. bifussispora             | EFCC 8260  | EF468807     | EF468855     | EF468747     |              |              |
| C. blackwelliae             | TBRC 7253  | MF140705     | MF140774     | MF140825     |              |              |
| C. blackwelliae             | TBRC 7254  | MF140704     | MF140773     | MF140824     |              |              |
| C. blackwelliae             | TBRC 7255  | MF140703     | MF140772     | MF140823     |              |              |
| C. blackwelliae             | TBRC 7256  | MF140702     | MF140771     | MF140822     |              |              |
| C. blackwelliae             | TBRC 7257  | MF140701     | MF140770     | MF140821     |              |              |
| C. ninchakispora            | EFCC 5197  | EF468820     | EF468868     | EF468760     |              |              |
| C. ninchakispora            | EFCC 5693  | EF468821     | EF468869     | EF468762     |              |              |
| C. ninchakispora            | EG5 38.165 | EF468846     | EF468900     | EF468795     |              |              |
| C. ninchakispora            | EG5 38.166 | EF468847     | EF468901     | EF468794     |              |              |
| C. ninchakispora            | NHJ 10627  | EF468822     | EF468870     | EF468763     |              |              |
| C. ninchakispora            | NHJ 10684  | EF468823     | EF468871     | EF468761     |              |              |
| Engyodontium aranearum      | CBS 309.85 | AF339526     | DQS22387     | DQS22341     |              |              |
| Gibellula longispora        | NHJ 12014  | EU369055     | EU369017     |              |              |              |
| G. pulchra                  | NHJ 10808  | EU369035     | EU369056     | EU369018     |              |              |
| G. ratticaudata             | ARSEF 1915 | DQS18777     | DQS22408     | DQS22360     |              |              |
| Gibellula sp.               | NHJ 5401   | EU369059     |              |              |              |              |
| Gibellula sp.               | NHJ 10788  | EU369036     | EU369058     | EU369019     |              |              |
| Gibellula sp.               | NHJ 13158  | EU369037     | EU369057     | EU369020     |              |              |
| Hevansia arachnophila       | NHJ 10469  | EU369031     | EU369047     | EU369008     |              |              |
| H. cinerea                  | NHJ 3510   | EU369048     | EU369009     |              |              |              |
| H. novoguineensis           | NHJ 4314   | EU369051     | EU369012     |              |              |              |
| H. novoguineensis           | NHJ 11923  | EU369032     | EU369052     | EU369013     |              |              |
| H. novoguineensis           | NHJ 13117  | EU369049     | EU369010     |              |              |              |
| H. novoguineensis           | NHJ 13161  | EU369050     | EU369011     |              |              |              |
| Hyperdermium pulvinatum     | PC. 602    | AF242353     | DQS27327     | DQS18746     |              |              |
| Lecanicillium aranearum     | CBS 726.73a| AF339537     | EF468887     | EF468781     |              |              |
| L. antillanum               | CBS 350.85 | AF339536     | DQS22336     | DQS22350     |              |              |
| L. fusiforme                | CBS 164.70 | AF339549     | EF468889     | EF468783     |              |              |
| L. psalliotae               | CBS 563.86 | AF339559     | EF468890     | EF468784     |              |              |
| Continued                   |            |              |              |              |              |              |
| Species                        | Strain no. | GenBank accession no. |
|-------------------------------|------------|-----------------------|
|                               |            | ITS       | SSU       | LSU       | RPB1     | TEF       |
| *L. psalliotae*               | CBS 532.81 | AF339560  | EF469096  | EF469067  |           |           |
|                               | CBS 101270 | EF469081  | EF469095  | EF469066  |           |           |
| *Pochonia chlamydosporia*     | CBS 103.65 | MH858504  |           |           |           |           |
| *Purpureocillium lilacinum*   | CBS 284.36 | FR775484  | EF468898  | EF468792  |           |           |
| *P. lilacinum*                | CBS 431.87 | EF468844  | EF468897  | EF468791  |           |           |
| *Samsoniella alboaurantium*   | CBS 240.32 | JF415979  | JN049895  | JF416019  |           |           |
| *S. alboaurantium*            | CBS 262.58 | MG665232  | QJ425685  |           |           |           |
| *S. aurantia*                 | TBRC 7271  | MF140728  | MF140791  | MF140846  |           |           |
|                               | TBRC 7272  | MF140727  | MF140817  | MF140845  |           |           |
| *S. inthanonensis*            | TBRC 7915  | MF140725  | MF140790  | MF140849  |           |           |
|                               | TBRC 7916  | MF140724  | MF140789  | MF140848  |           |           |
| *Pochonia chlamydosporia*     | CBS 103.65 | MH858504  |           |           |           |           |
| *Purpureocillium lilacinum*   | CBS 284.36 | FR775484  | EF468898  | EF468792  |           |           |
| *P. lilacinum*                | CBS 431.87 | EF468844  | EF468897  | EF468791  |           |           |
| *Samsoniella alboaurantium*   | CBS 240.32 | JF415979  | JN049895  | JF416019  |           |           |
| *S. alboaurantium*            | CBS 262.58 | MG665232  | QJ425685  |           |           |           |
| *S. aurantia*                 | TBRC 7271  | MF140728  | MF140791  | MF140846  |           |           |
|                               | TBRC 7272  | MF140727  | MF140817  | MF140845  |           |           |
| *S. inthanonensis*            | TBRC 7915  | MF140725  | MF140790  | MF140849  |           |           |
|                               | TBRC 7916  | MF140724  | MF140789  | MF140848  |           |           |
| *S. inthanonensis*            | TBRC 7270  | MF140723  | MF140788  | MF140847  |           |           |
| *Simplicillium aogashimaense* | JCM 18167  | AB604002  |           |           |           |           |
| *S. aogashimaense*            | JCM 18168  | AB604004  |           |           |           |           |
| *S. calcicola*                | LC5371     | KU746705  | KU74675   |           |           |           |
|                               | LC5586     | KU746706  | KU746752  |           |           |           |
| *S. chilense*                 | LC1342     | QJ410323  | QJ410321  |           |           |           |
| *S. chinense*                 | LC1345     | NR 155782 | QJ410322  |           |           |           |
| *S. cicadellidae*             | FY11011    | MN006243  | MN022271  | MN022263  |           |           |
| *S. cicadellidae*             | FY11012    | MN006244  | MN022272  | MN022264  |           |           |
| *S. coffeae*                  | COAD 2057  | MF066034  | MF066032  |           |           |           |
|                               | COAD 2061  | MF066035  | MF066033  |           |           |           |
| *S. cylindrosporum*           | JCM 18169  | AB603989  |           |           |           |           |
| *S. cylindrosporum*           | JCM 18170  | AB603994  |           |           |           |           |
|                               | JCM 18171  | AB603997  |           |           |           |           |
|                               | JCM 18172  | AB603998  |           |           |           |           |
|                               | JCM 18173  | AB603999  |           |           |           |           |
|                               | JCM 18174  | AB604005  |           |           |           |           |
|                               | JCM 18175  | AB604006  |           |           |           |           |
| *S. filiforme*                | URM 7918   | MH979338  | MH979399  |           |           |           |
| *S. formicicola*              | MFLUCC 18–1379 | MK766511 | MK765046 | MK82623 | MK926451 |           |
|                               | DL10041    | MN006241  | MN022269  |           |           |           |
| *S. formicicola*              | DL10042    | MN006242  | MN022270  |           |           |           |
| *S. lameliella*               | CBS 116.25 | AJ292393  | AF339552  | DQ522404  | DQ522356  |           |
|                               | KY00006    | AS78533   |           |           |           |           |
| *S. lameliella*               | UAMH 2055  | AF108471  |           |           |           |           |
|                               | UAMH 4785  | AF108480  |           |           |           |           |
| *S. lanosoniveum*             | CBS 101267 | AJ292395  | AF339553  | DQ522406  | DQ522358  |           |
| *S. lanosoniveum*             | CBS 704.86 | AJ292396  | AF339554  | DQ522405  | DQ522357  |           |
| *S. leptidoperorum*            | GY29131    | MN006246  | MN022273  | MN022265  |           |           |
|                               | GY29132    | MN006245  | MN022274  | MN022266  |           |           |
| *S. minatense*                | JCM 18176  | AB603992  | LC496893  |           |           |           |
| *S. minatense*                | JCM 18177  | AB603991  |           |           |           |           |
| *S. minatense*                | JCM 18178  | AB603993  | LC496894  |           |           |           |
| *S. obclavatum*               | CBS 31174  | AJ292394  | AF339517  | EF468798  |           |           |
| *S. obclavatum*               | JCM 18179  | AB604000  |           |           |           |           |
| *S. spumae*                   | JCM 39050  | LC496869  | LC496888  | LC496913  |           |           |
| *S. spumae*                   | JCM 39051  | LC496870  | LC496884  | LC496914  |           |           |
| *S. spumae*                   | JCM 39054  | LC496871  | LC496902  | LC496877  | LC496917  |           |
| *S. subtropicum*              | JCM 18180  | AB603990  | LC496895  |           |           |           |
| *S. subtropicum*              | JCM 18181  | AB603995  | LC496896  |           |           |           |
| *S. subtropicum*              | JCM 18182  | AB603996  |           |           |           |           |
| *S. subtropicum*              | JCM 18183  | AB604001  |           |           |           |           |
| *S. symposiophorum*           | JCM 18184  | AB604003  | LC496897  |           |           |           |
| Continued                      |            |           |           |           |           |           |
Table 2.  Taxa included in the phylogenetic analyses. Sequences generated in this study are shown in bold.

| Species                   | Strain no. | GenBank accession no. |
|---------------------------|------------|-----------------------|
|                           |            | ITS       | SSU       | LSU       | RPB1      | TEF       |
| S. coccinellidae          | DY101791   | MT453861  | MT453863  | MT453862  | MT471341  |
| S. coccinellidae          | DY101792   | MT453864  | MT453861  | MT457410  | MT471342  |
| S. hymenopterorum         | DY101691   | MT453848  | MT453849  | MT453850  | MT471344  | MT471337  |
| S. hymenopterorum         | DY101692   | MT453851  | MT453852  | MT453853  | MT471338  |
| S. neolepidopterorum      | DY101751   | MT453854  | MT453856  | MT453855  | MT471339  |
| S. neolepidopterorum      | DY101752   | MT453857  | MT453859  | MT453858  | MT471340  |
| S. scarabaeoidea          | DY101391   | MT453842  | MT453843  | MT453844  | MT471343  | MT471335  |
| S. scarabaeoida           | DY101392   | MT453845  | MT453847  | MT453846  | MT471336  |

Torriabulla wallacei        | CBS 101237 |           |           |           |           |           | 184967   | EF469102 | EF469073 |

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Author contributions
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Competing interests
The authors declare no competing interests.

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