The genus *Simplicillium*

De-Ping Wei¹²³⁴, Dhanushka N. Wanasinghe³⁵, Kevin D. Hyde²⁴, Peter E. Mortimer³, Jianchu Xu³⁵, Yuan-Pin Xiao²⁶⁷, Chitrabhanu S. Bhunjun²⁷, Chaiwat To-anun¹

¹ Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand ² Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand ³ Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Science, Kunming 650201, Yunnan, China ⁴ Mushroom Research Foundation, 128 M.3 Ban Pa Deng T. Pa Pae, A. Mae Taeng, Chiang Mai 50150, Thailand ⁵ World Agroforestry Centre, East and Central Asia, Kunming 650201, Yunnan, China ⁶ Engineering Research Center of Southwest Bio-Pharmaceutical Resources, Ministry of Education, Guizhou University, Guiyang, Guizhou Province, 550025, China ⁷ School of Science, Mae Fah Luang University, Chiang Rai, 57100, Thailand

Corresponding author: Peter E. Mortimer (petermortimer@mac.com)

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Abstract

*Simplicillium* species have a wide host range and an extensive distribution. Some species are associated with rusts, as well as other plant pathogenic fungi and play an important role in biological control. In this study, two specimens of *Simplicillium* were collected from Chiang Mai Province, Thailand. *Simplicillium formicae* sp. nov. was isolated from an infected ant and *S. lanosoniveum* from *Ophiocordyceps unilateralis* which is a new host record. Species were initially identified using ITS gene sequences and confirmed using morphology coupled with phylogenetic analyses of a combined nrLSU, nrSSU, TEF and RPB1 dataset. *Simplicillium formicae* differs from other species in the genus by the presence of flask-shaped synnemata and phialides with intercalary nodes. *Simplicillium lanosoniveum* resembles other collections of the species by its completely solitary, tapering phialides and globose to ellipsoidal conidia which adhere in a slimly head. A key to species of *Simplicillium* is also provided.

Keywords

new species, Thailand, ant fungi, taxonomy, phylogeny

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Introduction

Zare and Gams (2001) introduced *Simplicillium* to accommodate four taxa including the type species *S. lanosoniveum* and three other species, *S. lamellicola*, *S. obclavatum* and *S. wallacei*. *Simplicillium* species were historically placed in *Verticillium* sect. *Prostrata* which was described by Gams (1971) for prostrate conidiophore-producing species. Later, most of the species of *Verticillium* sect. *Prostrata* were reported as members in Clavicipitaceae, based on molecular data (including SSU, LSU and ITS sequences), whereas *Simplicillium* species consistently formed a monophyletic group apart from the other described taxa in this family (Zare et al. 2000; Gams and Zare 2001; Sung et al. 2001; Zare and Gams 2001). Recently, Clavicipitaceae was divided into three families, based on multi-gene phylogenetic analyses and *Simplicillium* was assigned to Cordycipitaceae (Hypocreales, Hypocreomycetidae, Sordariomycetes) (Sung et al. 2007; Maharachchikumbura et al. 2016; Wijayawardene et al. 2018). Zare and Gams (2008) excluded *Simplicillium wallacei* from *Simplicillium* and transferred it to *Lecanii- cillium* due to the basal position being closer to the latter genus than to the former genus in the cladogram of ITS data. Subsequently, ten species viz. *Simplicillium chinense* (Liu and Cai 2012), *S. aogashimaense*, *S. cylindrosporum*, *S. minatense*, *S. subtropicum*, *S. sympodiophorum* (Nonaka et al. 2013), *S. lanosoniveum var. tianjinensis* (Dong et al. 2014), *S. calcicola* (Zhang et al. 2017), *S. coffeanum* (Gomes et al. 2018) and *S. filiforme* (Crous et al. 2018) were restricted to *Simplicillium*, based on the phylogenetic analyses of ITS sequence data and strong morphological evidence. Its sexual-asexual connection has been established with *S. lanosoniveum* linked to a *Torrubiella* sp. (Zare and Gams 2001).

*Simplicillium* species have a wide distribution and are considered as mammal and plant-parasitic, symbiotic, entomopathogenic, fungicolous and nematophagous fungi, as they have a broad spectrum of hosts and substrates, such as insects, plants, rusts, nematodes, human nails, canine tissues and mushrooms, *Chroococcus* sp., soil, freshwater, marine and terrene environments (Zare and Gams 2001; Guo et al. 2012; Liu and Cai 2012; Dong et al. 2014; Liang et al. 2016; Sun et al. 2019). Several studies have been shown that *Simplicillium* species have a high ecological and economical value for biocontrol and bioactive compounds (Takata et al. 2013; Yan et al. 2015; Hyde et al. 2019). For example, *Simplicillium lanosoniveum* can be a phytopathogen, causing brown spots and lesions on flowers (Chen et al. 2008) or a mycoparasite on soybean rust (Ward et al. 2012; Gauthier et al. 2014) or a pathogen on aphids and other phytopathogens (Chen et al. 2017) or an anti- *Trichomonas vaginalis* agent (Scopel et al. 2013). *Simplicillium chinense* can be a biological control agent against plant parasitic nematodes (Zhao et al. 2013; Luyen 2017). *Simplicillium lamellicola* can suppress plant bacterial diseases and grey mould diseases of tomato (*Solanum lycopersicum*) and ginseng (*Panax ginseng*) (Dang et al. 2014; Shin et al. 2017). *Simplicillium obclavatum* has the ability to produce multiple xylanases and endoglucanases that have the potential to be used in biofuels, animal feed and food industry applications (Roy et al. 2013). Bioactive compounds with anti-fungal and anti-bacterial profiles and pharma-
The genus *Simplicillium* 71

ceutical exopolysaccharides have been isolated from *S. lano soniveum* (Yu et al. 2013; Fukuda et al. 2014; Xing et al. 2016; Dong et al. 2018). Linear and cyclic peptides with anti-fungal and anti-viral properties have also been discovered from the secondary metabolites of *S. obclavatum* (Liang et al. 2016, 2017).

Recent studies have shown that Thailand supports an amazing fungal diversity with numerous new species that have the potential for biotechnological application (Hyde et al. 2018, 2019). In this study, we introduce a novel species, *Simplicillium formicae* from northern Thailand and a new record of *S. lano soniveum* with evidence from a combination of molecular analyses and morphological characteristics to reserve a natural resource for future studies regarding biocontrol in the forestry, agricultural and pharmaceutical industries.

**Material and methods**

**Sample collection and isolation**

The Mushroom Research Centre (MRC) is a disturbed rainforest located in Chiang Mai Province, Thailand (Aung et al. 2008). The forest consists of various tall tree and lower shrubs. The climate of Chiang Mai is controlled by tropical monsoons and the weather is typically hot and humid with temperatures often close to or above 30 °C. Frequent rain and thunder showers usually last from June to late October (Chiang Mai Buddy website: https://chiangmaibuddy.com/welcome-to-chiang-mai/weather-and-climate/, accessed 26.8.2019). Two ant fungi were found anchored to the underside of two different shrubby leaves in the forest at the Mushroom Research Centre. These two fresh specimens; HKAS 102459 and HKAS 102447 were collected and placed in plastic containers and transported to the laboratory for subsequent study. Interestingly, the ant fungus HKAS 102447 was already dead and was colonised by a saprobic fungus. The isolate MFLUCC 18–1385 was separated from this saprobe which occurred on the surface of specimen HKAS 102447 via single spore isolation. The isolate MFLUCC 18–1379 was separated from specimen HKAS 102459 by directly cultivating the hyphae which covered the surface of the ant host. These two isolates were cultured with potato dextrose agar (PDA, 1% w/v peptone) and incubated at room temperature (25 °C).

**Morphological studies**

For long-term deposit, these two specimens were dried with allochroic silica gel to protect them from contamination of opportunistic fungi and to retain the informative taxonomic characters. The macro-morphological characters were observed with a stereoscope (Olympus SZ61) and the micro-morphological features were examined with a compound microscope (Nikon ECLIPSE Ni). Important characteristics such as myce-
lium, phialides and conidia were captured with a digital camera (Canon EOS 600D). Measurements of perithecia, synnemata, phialides and conidia were taken using the Tarosoft (R) Image Frame Work programme and the images used were processed with Adobe Photoshop CS3 Extended v. 10.0 (Adobe, San Jose, CA).

**DNA extraction, PCR amplification and sequencing**

DNA was extracted from fresh mycelium of isolates MFLUCC 18–1379 and MFLUCC 18–1385 and from stromal tissue of ant fungus HKAS 102447 (the host of isolate MFLUCC 18–1385) using a DNA extraction kit (Biospin Fungus Genomic DNA Extraction Kit, Bio Flux, China), following the instructions of the manufacturer. Extracted DNA was stored at 4 °C for use in regular work and duplicated at –20 °C for long-term storage. The internal transcribed spacer (ITS1-5.8S-ITS2, ITS) was amplified with primer ITS4 and ITS5 (White et al. 1990) and was used for individual gene phylogenetic analyses. The large subunit (LSU), small subunit rDNA (SSU), translation elongation factor 1-alpha gene (TEF1-α) and RNA polymerase II largest subunit 1 (RPB1) were also amplified as described in Wei et al. (2018) and used for multi-gene phylogenetic analyses. The PCR products were sent to Sangon Company, Kunming City, Yunnan Province, China for sequencing using the above primers. Newly generated sequences, used in the study, were submitted to GenBank to be assigned their accession numbers.

**Sequence alignments and phylogenetic analyses**

The raw sequences were verified with Finch TV version 1.4.0 (Mccredden 2016) and assembled with BioEdit v. 7.0.9.1 (Hall 1999). Sequence data were downloaded from GenBank based on BLAST searches of ITS sequences and with reference to the recent publications (Table 1). Most *Simplicillium* species are lacking protein-coding genes, but ITS sequences are available for all the species that are useful in understanding the intraspecific relationships within *Simplicillium* (Liu and Cai 2012, Nonaka et al. 2013, Dong et al. 2014 and Crous et al. 2018). Therefore, phylogenetic analyses, based on ITS regions, were generated throughout *Simplicillium* for the primary identification. Multi-gene phylogenetic analysis of the combined SSU, LSU, TEF and RPB1 sequences from representative species in Hypocreales was afterwards performed to confirm the taxonomic placements of our isolates.

The generated sequences of each gene region were aligned separately with representative sequences using MAFFT v. 7 web server (http://mafft.cbrc.jp/alignment/server) (Kuraku et al. 2013; Katoh et al. 2017). The uninformative gaps and ambiguous regions were manually removed and different gene regions were concatenated using BioEdit v. 7.0.9.1 (Hall 1999). The maximum Likelihood (ML) analyses was performed using RAxML-HPC2 on XSEDE (8.2.10) at CIPRES Science Gateway V. 3.3 (https://www.phylo.org/portal2/home.action), with default setting, except the boot-
| Taxon                                      | Voucher no. | Host/substrate | SSU rRNA | LSU rRNA | tef1 | rPrb1 | Reference                  |
|--------------------------------------------|-------------|----------------|----------|----------|------|-------|----------------------------|
| Acanthonyces taberculata                   | BCC 8105    | Scale insect   | DQ522537 | DQ518752 | DQ522317 | DQ522363 | Spatafora et al. (2007)    |
| Acherontia badia                           | BCC 7957    | Scale insect   | DQ522538 | DQ518753 | DQ522318 | DQ522364 | Spatafora et al. (2007)    |
| Acherontia placenta                        | GAM         | Panicaceae sp. | AY545723 | AY545727 | AY489610 | AY489643 | Spatafora et al. (2007)    |
| Balaninus henningsiana                     | AEG 94-2    | Poaceae        | AF543764 | AF543788 | DQ522319 | DQ522365 | Spatafora et al. (2007)    |
| Balaninus pilulaiformis                    | ATCC 26019  | Poaceae        | DQ522539 | U17402   | DQ522320 | DQ522366 | Spatafora et al. (2007)    |
| Claviceps fusiformis                       | ATCC 13892  | Poaceae        | U32401   | U47826   | DQ522321 | DQ522367 | Spatafora et al. (2007)    |
| Claviceps purpurea                         | ARSEF 5413  | Irregular sp.  | AY184979 | AY184968 | DQ522351 | DQ522397 | Spatafora et al. (2007)    |
| Cordyceps farinosa                         | ARSEF 5689  | Scabellidae pupa | AF339574 | AF339524 | DQ522335 | DQ522380 | Spatafora et al. (2007)    |
| Cordyceps tentipes                         | OSC 111005  | Lepidoptera pupa | DQ522558 | DQ518773 | DQ522348 | DQ522394 | Spatafora et al. (2007)    |
| Drechmeria balanoides                      | CBS 250.82  | Nematoda       | AF339588 | AF339539 | DQ522342 | DQ522388 | Spatafora et al. (2007)    |
| Drechmeria gunnii                          | OSC 76404   | Lepidoptera larva | AF339572 | AF339522 | AY489616 | AY489650 | Spatafora et al. (2007)    |
| Drechmeria sinensis                        | CBS 567.95  | Nematoda       | AF339594 | AF339545 | DQ522343 | DQ522389 | Spatafora et al. (2007)    |
| Engydontium araneaeum                      | CBS 309.85  | Spider         | AF339576 | AF339526 | DQ522341 | DQ522387 | Spatafora et al. (2007)    |
| Epichloe typhina                           | ATCC 56429  | Festucca rubra | U32405   | U17396   | AF543777 | AY489653 | Spatafora et al. (2007)    |
| Hypocrella necrinitoides                   | GJS 89-104  | Scale insect   | U32409   | U47832   | DQ522347 | DQ522393 | Spatafora et al. (2007)    |
| Hypocrella schizantacbyi                   | BCC 14123   | Scale insect   | DQ522557 | DQ518771 | DQ522346 | DQ522392 | Spatafora et al. (2007)    |
| Lecanicillium antilamellum                 | CBS 350.85  | Agar           | AF339585 | AF339536 | DQ522350 | DQ522396 | Spatafora et al. (2007)    |
| Lecanicillium lecanii                      | CBS 101247-IMI | 304807 | Lepidoptera | AY184967 | EF460973 | EF460102 | Zare and Gams (2008); Kouvelis et al. (2008) |
| Lecanicillium wallacei                     | CBS 101237-IMI | 331549 | Lepidoptera | AY184967 | EF460973 | EF460102 | Zare and Gams (2008); Kouvelis et al. (2008) |
| Metarhizium ehlmydoporia                  | CBS 101244  | Mollusca       | DQ522544 | DQ518758 | DQ522327 | DQ522372 | Spatafora et al. (2007)    |
| Metarhizium ehlmydoporia                  | ARSEF 5714  | Lepidoptera    | AF543763 | AF543787 | AF543775 | DQ522383 | Spatafora et al. (2007)    |
| Metapochonia goniodes                      | CBS 891.72  | Nematoda       | AF339599 | AF339550 | DQ522354 | DQ522401 | Spatafora et al. (2007)    |
| Metarhizium album                          | ARSEF 2082  | Aegina sp.     | DQ522560 | DQ518775 | DQ522352 | DQ522398 | Spatafora et al. (2007)    |
| Metarhizium flavoearise                    | ARSEF 2037  | Nilaparvata lugens | AF339580 | AF339531 | DQ522353 | DQ522400 | Spatafora et al. (2007)    |
| Metarhizium majus                          | ARSEF 3145  | Oryctes rhinoceros | AF339579 | AF339530 | AF543774 | DQ522399 | Spatafora et al. (2007)    |
| Taxon                  | Voucher no. | Host/substrate | SSU rRNA | LSU rRNA | tef1 | tεp1 | Reference                        |
|-----------------------|-------------|----------------|----------|----------|------|------|----------------------------------|
| *Myriogenospora*      | AEG 96-32   | *Andropogon*   | AY489701 | AY489733 | AY489628 | AY489665 | Spatafora et al. (2007)          |
| *Ophiocordyceps*      | OSC 128580  | Coleoptera     | DQ522543 | DQ518757 | DQ522326 | DQ522371 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | ARSEF 5498  | Coleoptera     | DQ522541 | DQ518755 | DQ522323 |          | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 128576  | Coleoptera     | DQ522542 | DQ518756 | DQ522324 | DQ522369 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 128577  | Ant            | DQ522546 | DQ518760 | DQ522329 | DQ522374 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 128578  | Ant            | DQ522556 | DQ518770 | DQ522345 | DQ522391 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 110993  | Scarabaeidae   | DQ522548 | DQ518762 | DQ522331 | DQ522376 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 110994  | Stink bug      | DQ522549 | DQ518763 | DQ522333 | DQ522378 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 110995  | *Phyllophaga*  | DQ522550 | DQ518764 | DQ522334 | DQ522379 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 110998  | *Wasp*         | DQ522551 | DQ518765 | DQ522336 | DQ522381 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 111000  | Elateridae grub| DQ522552 | DQ518766 | DQ522337 | DQ522382 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | OSC 128574  | Ant            | DQ522554 | DQ518768 | DQ522339 | DQ522385 | Araújo et al. (2018)            |
| *Ophiocordyceps*      | ARSEF 5365  | Diptera larva  | DQ522555 | DQ518769 | DQ522340 | DQ522386 | Spatafora et al. (2007)         |
| *Rosigrophthora*       | CBS 101437  | Rotifer         | AF339584  | AF339535  | AF543776  | DQ522402 | Spatafora et al. (2007)         |
| *Simplicillium*        | LC5586 =    | Calcare         | KX855252  |          |          |          | Spatafora et al. (2007)         |
| *Simplicillium*        | CGMCC3.17943|                | KU746752  |          |          |          | Spatafora et al. (2007)         |
| *Simplicillium*        | CBS 116.25  | *Agaricus*     | AF339601  | AF339552  | DQ522356 | DQ522404 | Spatafora et al. (2007)         |
| *Simplicillium*        | CBS 704.86  | *Hemileia*     | AF339602  | AF339553  | DQ522358 | DQ522406 | Spatafora et al. (2007)         |
| *Simplicillium*        | CBS 311.74  | Air above       | AF339567  | AF339517  | EF468798  |          | Spatafora et al. (2007)         |
| *Tolypocladium*        | OSC 110990  | *Elaphomyces*  | DQ522545 | DQ518759 | DQ522328 | DQ522373 | Spatafora et al. (2007)         |
| *Tolypocladium*        | OSC 110991  | *Elaphomyces*  | DQ522547 | DQ518761 | DQ522330 | DQ522375 | Spatafora et al. (2007)         |
| *Torrubiella*          | ARSEF 1915  | *Euophrys*     | DQ522562 | DQ518777 | DQ522360 | DQ522408 | Spatafora et al. (2007)         |
| *Verticillium*         | CBS 384.81  | *Hemileia*     | AF339596  | AF339547  | DQ522361 | DQ522409 | Spatafora et al. (2007)         |

strap iterations were set to 1,000 and the substitution model set to GTR+GAMMA + I (Miller and Blair 2009). Maximum Parsimony analysis (MP) was performed by PAUP v. 4.0b10 (Swofford 2002) with the heuristic search option and Tree-Bisection-Reconnection (TBR) branch-swapping algorithm for 1000 random replicates. Branches that have a minimum branch length of zero were collapsed. Gaps were treated as “missing” and starting tree(s) were generated via stepwise addition (Hillis and Bull 1993). Tree Length [TL], Consistency Index [CI], Retention Index [RI], Rescaled Consistency Index [RC] and Homoplasy Index [HI]) were calculated for all parsimonious trees. For Bayesian analysis, the best models of each gene were selected under Akaike Information Criterion (AIC) employing MrModeltest v. 2.3 (Nylander et al. 2008) and
PAUP v. 4.0b10 (Ronquist and Huelsenbeck 2003). Bayesian analysis was performed using MrBayes v. 3.1.2 (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002) to evaluate posterior probabilities (BYPP) with the Markov Chain Monte Carlo sampling (MCMC) method. Trees were sampled and printed to output at every 1000 generations. The first 25% of sampled trees were discarded as part of a burn-in procedure, the rest of the trees were used to create the consensus tree and the average standard deviation of split frequencies was set as 0.01.

Phylogenetic trees were visualised with FigTree v1.4.0 (Rambaut 2006) and edited in Microsoft PowerPoint, then saved as a PDF format and finally altered to JPG format using Adobe Illustrator CS6 (Adobe Systems Inc., United States). The finalised alignments and trees were submitted in TreeBASE (http://www.treebase.org/), with submission ID 24238 (ITS) and 24240 (multi-gene).

**Results and discussion**

**Phylogenetic analysis**

The combined four gene dataset comprised 60 taxa from three families (Cordycipitaceae, Ophiocordycipitaceae and Clavicipitaceae) (Table 1) with *Cosmospora coccinea*, *Nectria cinnabarina*, *Ophionectria trichospora* and *Viridispora diparietispora* as the outgroup taxa. The RAxML analysis of the combined dataset yielded a best scoring tree (Figure 1) with a final ML optimisation likelihood value of $-39792.595439$. The alignment comprised 3469 total characters including gaps, of which 2077 were constant, 338 variable characters parsimony-uninformative and 1054 characters parsimony-informative. The Kishino-Hasegawa (KH) test showed $CI = 0.281$, $RI = 0.527$, $RC = 0.148$ and $HI = 0.719$. The matrix had 1655 distinct alignment patterns, with 6.42% undetermined characters or gaps. Estimated base frequencies were as follows: $A = 0.241091$, $C = 0.260362$, $G = 0.272837$, $T = 0.225710$; substitution rates $AC = 0.985172$, $AG = 2.843760$, $AT = 0.887714$, $CG = 0.898140$, $CT = 6.284116$, $GT = 1.000000$; gamma distribution shape parameter $\alpha = 0.585080$. MrModeltest v. 2.3 imply that GTR+I+G is the best-fit model for LSU and RPB1, SYM+I+G for SSU and TEF sequences.

The ITS dataset comprised 49 taxa from all *Simplicillium* species that are currently available in GenBank (Figure 2) with *Cordyceps militaris* (CBS178.59) (Cordycipitaceae, Hypocreales) as the outgroup taxon. The RAxML analysis of the ITS dataset yielded a best scoring tree (Figure 2) with a final ML optimisation likelihood value of $-3155.597177$. The alignment comprised 570 total characters including gaps, of which 346 were constant, 43 variable characters parsimony-uninformative and 181 characters parsimony-informative. The Kishino-Hasegawa (KH) test showed $CI = 0.681$, $RI = 0.856$, $RC = 0.583$ and $HI = 0.319$. The matrix had 283 distinct alignment patterns, with 6.45% undetermined characters or gaps. Estimated base frequencies were as follows: $A = 0.232003$, $C = 0.283823$, $G = 0.254774$, $T = 0.229400$; substitution rates
Figure 1. Phylogram generated from maximum likelihood analysis based on combined SSU, LSU, TEF and RPB1 sequence data. Bootstrap values for maximum likelihood (ML, left) and maximum parsimony (MP, right) equal to or greater than 50% and Bayesian posterior probabilities (BYPP, middle) equal to or greater than 0.90 are placed nearby the note. The newly generated sequences are indicated in red bold.

AC = 2.623562, AG = 2.645665, AT = 2.248749, CG = 1.653083, CT = 5.842034, GT = 1.000000; gamma distribution shape parameter $\alpha = 0.980038$. MrModeltest v. 2.3 imply that GTR+I+G is the best-fit model for ITS sequences.
The genus *Simplicillium*

**Figure 2.** Phylogram generated from maximum likelihood analysis based on ITS sequence data. Bootstrap values for maximum likelihood (ML, left) and maximum parsimony (MP, right) equal to or greater than 50% and Bayesian posterior probabilities (BYPP, middle) equal to or greater than 0.90 are placed nearby the branches, respectively. The newly generated sequences are indicated in red bold and the type species are highlighted in black bold.

The multi-gene phylogenetic analyses showed that our isolates MFLUCC 18-1379 and MFLUCC 18–1385 grouped with the remaining *Simplicillium* species with strong support (100% ML, 1.00 BYPP, 100% MP, Figure 1) in Cordycipitaceae. The host of isolate MFLUCC 18–1385 grouped with *Ophiocordyceps unilateralis* (OSC...
De-Ping Wei et al. / MycoKeys 60: 69–92 (2019)

78

128574) in Ophiocordycipitaceae with a significant statistical support (100% ML, 1.00 BYPP, 100% MP, Figure 1). In the individual ITS-based phylogenetic tree, the isolate MFLUCC 18-1379 constituted a close affiliation to Simplicillium obclavatum with moderate bootstrap support (68% ML, 0.93 BYPP, 87% MP, Figure 2, clade 2). The fungal isolate MFLUCC 18-1385 grouped with the remaining Simplicillium lano-soniveum strains with 85% ML, 0.99 BYPP and 67% MP support (Figure 2, clade 1).

Taxonomy

Simplicillium W. Gams & Zare, Nova Hedwigia 73(1-2): 38 (2001)

Hyperparasitic on rusts or parasitic on nematodes or occurring in soil. Asexual morph: Mycelium thin, hyaline, septate, branched, smooth-walled. Phialides arising from prostrate aerial hyphae or rope-like and flask-shaped synnemata, typically solitary, rarely in whorls of 2–3, gradually tapering towards the apex, elongate, slender, smooth-walled, phialidic. Conidia hyaline, oval, spindle-shaped, cylindrical, subglobose to ellipsoidal, fusoid to filiform, straight to curved, smooth-walled. Conidia commonly form in small globose heads, sometimes in branched, unbranched, zigzag or imbricate chains, occasionally in sympodial proliferation with cylindrical conidium-bearing denticles. Colonies of species in this genus are usually fast growing, reaching 10–38 mm within 10 days on PDA, white, reverse brownish-cream to pale yellow, margin entire, cottony, fluffy or floccose. Some species produce yellow or orange pigment. Crystals are commonly present in the agar. Sexual morph: Torrubiella (Zare and Gams 2001; Liu and Cai 2012; Nonaka et al. 2013; Dong et al. 2014; Gomes et al. 2018; Zhang et al. 2017).

In this study, we introduce a new species and a new host species as described below.

Simplicillium formicae D.P. Wei & K.D. Hyde, sp. nov.

Index Fungorum number: IF556432
Facesoffungi number: FoF 05813

Figure 3, 4

Etymology. the epithet refers to its host–ant.

Holotype. HKAS 102459; living culture: MFLUCC 18–1379.

Description. Parasitic on ant (Formicidae). Asexual morph: Hyphomycetous. Mycelium rarely septate, hyaline, smooth-walled, covering the whole body of the ant host. Synnemata 250–350 × 65–100 (\(\bar{x} = 300 \times 86, n = 10\) μm, forming at the head region of ant host in circular arrangement, flask-shaped, hyaline to yellowish, composed of dense hyphae, somehow curved. Phialides 25–100 × 0.5–1.5 (\(\bar{x} = 49 \times 1.1, n = 20\) μm, arising from procumbent hyphae or synnemata, blastic, enteroblastic, phialidic, monophialidic, discrete, terminal, unbranched, solitary, aseptate, hyaline, smooth-walled, slender, occasionally a swollen node present. Conidia 2–3.5 × 1.5–2.5 (\(\bar{x} = 2.6 \times 2, \)
The genus *Simplicillium*

Figure 3. *Simplicillium formicae* (from HKAS 102459, holotype) a superficial hyphae associated with the ant host b–e flask-shaped synnemata f–k phialides bearing conidia l–p conidia. Scale bars: 1000 µm (a); 500 µm (b); 100 µm (d); 30 µm (e, f); 15 µm (j, k); 10 µm (l–p) (e stained with cotton blue solution).
Figure 4. *Simplicillium formicae* (MFLUCC 18–1379, ex-type living culture) a upper and reverse view of cultures on PDA after 30 days e–g phialides indicated with black arrow c, d, h–j conidial mass on the tip of phialides k–m conidia. Scale bars: 10 µm (c, d, f, g); 20 µm (e); 3 µm (h–j); 1 µm (k–m) (e–j stained with cotton blue solution).

n = 30) µm, globose to ellipsoidal, hyaline, one-celled, smooth-walled, round at both ends, adhering in slimy head on the tip of phialides. **Sexual morph**: Undetermined.

**Culture characteristics.** The colonies were rapid-growing on PDA medium, reaching a diameter of 2.5–3 (x = 2.6, n = 9) cm, in 13 days at 22 °C, entire margin, circu-
The genus *Simplicillium*

The genus *Simplicillium* lar, velvety and white from above, with radial crack and primrose-yellow on reverse. *In vitro*, *Synnemata* absent. *Phialides* 25–75 × 0.4–0.6 (\(\bar{x} = 50 \times 0.55, n = 10\)) \(\mu\)m, arising from procumbent hyphae, blastic, enteroblastic, phialidic, discrete, terminal, unbranched, solitary, aseptate, hyaline, smooth-walled, relatively slender and long. *Conidia* 1.5–3 × 1.5–2.5 (\(\bar{x} = 2.3 \times 1.7, n = 100\)) \(\mu\)m, hyaline, globose to ellipsoidal, aseptate, smooth-walled, slightly guttulate, adhering in slimy head on the tip of phialides.

**Material examined.** THAILAND, Chiang Mai Province, Mushroom Research Centre, on an adult ant, 1 April 2018, *Deping Wei*, MRC18040102 (*holotype*: HKAS 102459; *ex-type living culture*: MFLUCC 18–1379). Sequences generated from this strain have been deposited in GenBank with accession numbers: SSU = MK765046, LSU = MK766512, ITS = MK766511, TEF = MK926451, RPB1 = MK882623.

**Note.** Isolate MFLUCC 18–1379 has a close phylogenetic relationship with *Simplicillium obclavatum*, based on ITS sequence analysis. The new isolate is similar to *Simplicillium obclavatum* in terms of shape and dimensions of the conidia with slender phialides tapering towards the apex. However, they have a different conidial arrangement, by *Simplicillium obclavatum* having short-imbricate chains, whereas the new fungus has subglobose to globose head. There are numerous synnemata in a circular arrangement which can be observed in our isolate and those are absent in *Simplicillium obclavatum*. The comparisons of ITS sequences between our isolate MFLUCC 18–1379 and ex-type strain of *Simplicillium obclavatum* (CBS 311.74) show 23 bp differences within 550 bp (4.2%). Thereby, we identify our isolates as a new species according to Jeewon and Hyde (2016).

*Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams, *Nova Hedwigia* 73(1–2): 39 (2001)

Facesoffungi number: FoF 05814

Index Fungorum number: 532459

Figure 5

*Cephalosporium lanosoniveum* J.F.H. Beyma, Antonie van Leeuwenhoek 8: 121 (1942) (Basionym)

**Ex-type.** Netherlands, on hair of *Cibotium schiedei* in greenhouse, 1942, F.H. van Beyma, CBS123.42.

**Description.** Saprophytic on *Ophiocordyceps unilateralis*. *Asexual morph*: Hyphomycetous. Mycelium aseptate, hyaline, smooth-walled. *Phialides* 20–40 × 1.1–2 (\(\bar{x} = 30 \times 1.6, n = 20\)) \(\mu\)m, arising from the prostrate mycelium, blastic, enteroblastic, phialidic, monophialidic, discrete, terminal, aseptate, hyaline, smooth-walled, solitary, tapering toward the apex. *Conidia* 2–4.5 × 1–3 (\(\bar{x} = 3 \times 1.8, n = 60\)) \(\mu\)m, hyaline, amerospores, globose to ellipsoidal, smooth-walled, adhering in globose to ellipsoidal head at the apex of phialides. *Sexual morph*: Undetermined.

**Culture characters.** The colonies on PDA medium were rapid-growing, reaching a diam. of 5.5 cm in 30 days at 22 °C, white, entire margin, velvety, with radial cracks and primrose-yellow on the reverse.
Figure 5. *Simplicillium lanosoniveum* (a–f from HKAS 102447, g–r from MFLUCC 18–1385) a host (*Ophiocordyceps unilateralis*); b, c hyphae associated with host indicated with black arrows g, h upper and reverse view of cultures on PDA after 40 days incubation i–l conidial mass on the tip of phialides m–o phialides bearing conidia p–r conidia. Scale bars: 15 µm (i–m); 10 µm (d–f, n, o); 3 µm (p–r) (i, l–n stained with cotton blue solution).
Host and distribution: Saprophytic on fungi, endophytic or symbiotic or pathogenic on plant, parasitic on rust, nematode and insect, occurring on soil, animal hair or human bronchoalveolar lavage fluid, with a cosmopolitan distribution (see Table 2).

**Material examined.** THAILAND, Chiang Mai Province, Mushroom Research Centre, on *Ophiocordyceps unilateralis*, 19 February 2018, Deping Wei, MRC18021901 (HKAS 102447; living culture: MFLUCC 18–1385). Sequences generated from this strain have been deposited in GenBank with accession numbers: SSU = MK752791, LSU = MK752849, ITS = MK752683, TEF = MK926450, RPB1 = MK882622.

**Note.** Our isolate MFLUCC 18–1385 colonised on a decayed *Ophiocordyceps unilateralis* with white hyphae. In a thorough examination of the *Ophiocordyceps unilateralis* host, we found the phialides and conidia of our isolate grown on the surface of the host (Figure 5). Phylogenetically, our isolate grouped with the strains of *Simplicillium lanosoniveum* with high bootstrap support (85% ML, 0.99 BYPP, 67% MP, Figure 2). The nucleotides comparison between our isolate and the type strain of *Simplicillium lanosoniveum* (CBS123.42) showed only 5 bp differences out of 539 in the ITS region. This evidence proves that our isolate is a strain of *S. lanosoniveum*, according to Jeewon and Hyde (2016). Morphologically, it resembles *S. lanosoniveum* with solitary phialides without verticillate branches and conidia adhering on a slimy head. Most of the previous descriptions of this species were given in hand-drawings and scanning electron microscopy (SEM) patterns (Zare and Gams 2001; Ward et al. 2012; Gauthier et al. 2014). *Simplicillium lanosoniveum* has been reported from *Enhalus acoroides* (seagrass) in Trang Province, Thailand. In this study, we introduce our isolate MFLUCC 18–1385 as a new host record of *Simplicillium lanosoniveum* from *Ophiocordyceps unilateralis* and provide the updated morphological features for a better understanding of this species. *Simplicillium lanosoniveum* has been frequently reported as a hyperparasite of rust and plant pathogenic fungi. Therefore, this species has a high potential of being a natural source of microbial agents against microbiological diseases in commercial agriculture (Baiswar et al. 2014; Berlanga-Padilla et al. 2018). At first, we included all available sequences of *S. lanosoniveum* from GenBank in the individual gene tree. Some strains did not group with other strains but distributed throughout the genus in primary analyses (data not shown), so we excluded those strains from the final phylogenetic analysis. Most of the reported strains of *S. lanosoniveum*, including the invalid strains, are listed in Table 2 to show their distribution and host range, as well as the sequence data availability.

*Ophiocordyceps unilateralis* (Tul. & C. Tul.) Petch, *Trans. Br. mycol. Soc.* 16(1): 74 (1931)
Index Fungorum number: 281145;
Facesoffungi number: FoF 05815
Figure 6

**Description.** Parasitic on ants (*Formicidae*). **Sexual morph:** Stromata up to 14 mm in length, 0.5 mm wide in the broadest part, cylindrical, brown, slightly tapering towards the apex, single, piercing through the dorsal neck region of the ant host.
### Table 2. Distribution, host and available sequence data of *Simplicillium lanosoniveum* strains.

| Species                  | Strain no. | Host and habitat                  | Origin       | Available gene region | Morphological description | Reference                      |
|--------------------------|------------|-----------------------------------|--------------|-----------------------|----------------------------|---------------------------------|
| *S. lanosoniveum*        | CBS123.42  | Hair of *Cibotium schiedei* (Plant)| Netherland   | ITS, LSU              |                            | GenBank; Zare and Gams (2001)  |
|                          | Cg0701     | *Salvia molesta* (Plant)          | Taiwan       | ITS                   |                            | Chen et al. (2008)             |
|                          | PSU-ES104  | *Enhalus acuroides* (Plant)       | Trang Province, Thailand | ITS                  |                            | Supaphon et al. (2014)         |
|                          | CBS 531.72 | *Salvia rotundifolia* (Plant)     | USA          | ITS                   |                            | Zare and Gams (2001)           |
|                          | Tr3        | *Salvia milliorhiza* (Plant)      | China        | ITS                   |                            | GenBank                        |
|                          | YLAC-5     | Endophytic on *Inula aconitum* (Plant) | China      | ITS                   |                            | GenBank                        |
|                          |            | Endophytic on seaweed (Plant)     | India        | SSU                   |                            | GenBank                        |
|                          | E1, E3, E5 | Endophytes of *Sophora alopecuroides* (Plant) | Ningxia, China | SSU, ITS             |                            | Yu et al. (2013)               |
|                          | GA-B1      | *Grewia asiatica* (Plant)         | Shivalik region, Jammu, India | SSU   |                            | GenBank                        |
|                          | IMI 303103b| *Hemileia vastatrix* (Rust)       | Colombia     | ITS, SSU              |                            | Zare and Gams (2001), Kouvelis et al. (2008) |
|                          | AMH 9654 | Rust pustules on leaves of *Elaeagnus* sp. | India | LSU, ITS              |                            | Baiswar et al. (2014)          |
|                          | D082307-2A | Soybean rust                      | Louisiana    | ITS                   |                            | Ward et al. (2011)             |
|                          | vee-02     | Rust of *Elaeagnus latifolia*     | India        | ITS                   |                            | GenBank                        |
|                          | vee-01     | Rust of *Elaeagnus latifolia*     | India        | ITS                   |                            | GenBank                        |
|                          | CBS 704.86 | *Hemileia vastatrix* (Rust)       | Venezuela    | ITS, SSU, LSU, TEF, RPB1, RPB2, ATP |                            | GenBank; Zare et al. (2000)     |
|                          | S-599      | *Coleosporium plumeriae* (Rust)   | Campos dos Goytacazes, GJ, Brazil | ITS   |                            | Berlanga-Padilla et al. (2018)  |
|                          | D082307-2A-GFP15 | *Phakopsora pachyrhizi* (Rust) | Florida, USA | ITS                   |                            | Gauthier et al. (2014)          |
|                          | HKAS 102447| *Ophiocordyceps uninucleatus* (Fungi) | Chiang Mai, Thailand | SSU, LSU, ITS, TEF, RPB1 |                            | This study                     |
|                          | TLY001     | *Pseudococcus pentagona* (Insect) | Shanxi Province, China | ITS |                            | Wang et al. (2016)             |
|                          | SSBG2      | *Coccus hesperiidum* (Insect)     | The South-Siberian Botanical Garden, Russia | ITS |                            | Skaptsov et al. (2017)          |
|                          | TAMA 173   | *Aphiside a* (Insect)             | Ibaraki, Japan | ITS                   |                            | Fukuda et al. (2014)           |
|                          | CHE-CNRCB 373 | *Diaphorina citri* (Insect)      | Colima, USA  | ITS                   |                            | Berlanga-Padilla et al. (2018)  |
|                          | ARSEF 8822 | Culicid (Insect)                  | Tanzania     | ITS                   |                            | Hubner-Campos et al. (2013)    |
|                          | ARSEF7550  | Coccoidea (Insect)                | Brazil       | TEF                   |                            | GenBank                        |
|                          | 1T9BA      | Tick (Insect)                     | New York, USA | ITS                   |                            | Greengarten et al. (2011)      |
|                          | Btab03     | *Bemisia tabaci* (Insect)         | South Korea  | ITS                   |                            | GenBank                        |
|                          | 113-8      | Mosquitoes (Insect)               | Japan        | ITS                   |                            | Ishii et al. (2015)            |
| Species             | Strain no. | Host and habitat                  | Origin     | Available gene region | Morphological description | Reference                |
|---------------------|------------|-----------------------------------|------------|-----------------------|---------------------------|---------------------------|
| *S. lanosoniveum*   | 7S         | *Heterodera schachtii* (Nematode) | Iran       | ITS                   |                           | GenBank                   |
|                     |            | Hair of giant panda (Animal)      | China      | ITS                   |                           | GenBank                   |
|                     | 2502       | Bronchoalveolar lavage fluid (Human) | China      | ITS                   |                           | GenBank                   |
|                     | 41559-3    | Cave and mine                     | New York State, USA | ITS, LSU       |                           | GenBank                   |
| CBS 321.72          |            | Malaysia                          | Malaysia   | SSU, LSU, ITS         |                           | GenBank; Summerbell et al. (2011) |
| CBS 322.72          |            | Malaysia                          | Malaysia   | ITS                   |                           | GenBank                   |

Note: ‘√’ means related data are available. The strains collected from Thailand are indicated with **black bold**.

![Image of Ophiocordyceps unilateralis](image)

**Figure 6. Ophiocordyceps unilateralis** (from HKAS 102447) **a** stroma emerging from host **b** ascomata on stroma **c** host (Formicidae) **d, e** decayed perithecia. Scale bars: 500 µm (**b, c**); 300 µm (**d**); 100µm (**e**).

*Ascomatal cushion* hemisphere, up to 1.2 mm in diam., laterally attaching to the erect stroma stalk, dark brown, with ostioles protruding from the cushions. *Perithecia* 200–400 × 50–120 (x̄ = 294 × 81, n = 10) µm, sub-immersed, flask-shaped. *Asci* and ascospores were too old to observe their features. **Asexual morph:** Undetermined.
Note. This collection was already decayed and was colonised by other fungi which we introduced as a new host record of *Simplicillium lanosoniveum* from Thailand. The outline of this specimen was intact, while its asci and ascospores were too old to analyse. We retrieved DNA through direct sequencing from the stromal tissue.

Sequences generated from this specimen have been deposited in GenBank with accession numbers: SSU = MK752759, LSU = MK752812, ITS = MK752874. The herbarium material is deposited at KUN herbarium, Yunnan Province, China. In the multi-gene phylogenetic tree, this collection groups with *Ophiocordyceps unilateralis* (OSC 128574) with a strongly supported bootstrap value (100% ML, 1.00 BYPP, 100% MP, Figure 1). Therefore, we identify this collection as *O. unilateralis*, based on its morphologic features and molecular evidence.

Key to accepted species of *Simplicillium*

1a Conidia formed in sympodia ................................................. *S. sympodiophorum*
1b Conidia solitary, borne on the tip of phialides ...................... *S. calcicole*
1c Conidia aggregate in chains ..................................................2
1d Conidia aggregate in subglobose to ellipsoidal heads ...............3
1e Conidia aggregate in globose heads ........................................4
2a Conidia 2.5–3.5 × 1–2 μm, obclavate to ellipsoidal, formed in short imbricate chains ................................................................. *S. obclavatum*
2b Conidia 3.5–5.0 × 1.0–1.5 μm, oval, ellipsoidal or cylindrical, formed in vertical chains ............................................................. *S. chinense*
2c Conidia 7.2–12.5 × 1 μm, long, fusiform to short filiform, hyaline, straight to curved, formed in vertical chains ...................... *S. filiforme*
3a Phialides 15–50 × 0.7–1.0 μm, colonies light to dark-brown reverse on PDA, usually with yellow pigment diffusing into the agar ........ *S. lamellicola*
3b Phialides 11–44 (–70) × 1.0–2.4 μm, colonies cream-coloured reverse on PDA, no diffused pigment ........................................... *S. coffeanum*
4a Present flask-shaped synnemata .............................................5
4b Synnemata absent ....................................................................5
5a Conidia cylindrical ....................................................................6
5b Conidia globose to subglobose or ellipsoidal ............................7
6a Phialides 23–53 × 1.2–2.0 μm, long ........................................... *S. cylindrosporum*
6b Phialides 17–32 × 1.2–2.0(–2.5) μm, short .................................. *S. aogashima*
7a Phialides 35–75 × 1.2–3.0 μm, conidia 4.5–6.0 × 2.5–3.5 μm, colonies light yellow to deep tawny in reverse view on PDA ... *S. lanosoniveum var. tianjinensis*
7b Phialides 15–39 × 0.7–1.9 μm, conidia 1.5–3 × 0.7–1.3 μm, colonies brownish-cream to pale yellow reverse on PDA............. *S. lanosoniveum*
7c Phialides 11–31(–47) × 1.0–1.7 μm, conidia 2.0–3.5 × 1.8–2.5(–2.8) μm, colonies brown reverse on PDA ................................................................ *S. subtropicum*
7d Phialides (15–)20–42(–50) × 1.0–2.3 μm; conidia 2.3–4.0(–4.5) × 1.5–3.3 μm, colonies brownish-orange to brown reverse on PDA.............. *S. subtropicum*
Conclusion

A new species Simplicillium formicae and a new host record species Simplicillium lanosoniveum from Ophiocordyceps unilateralis were introduced, based on phylogenetic analyses and morphological evidence. The host and distribution of S. lanosoniveum was summarised and a key to Simplicillium was provided.

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