Multigene phylogeny and morphology reveal
*Ophiocordyceps hydrangea* sp. nov. and *Ophiocordyceps bidoupensis* sp. nov. (Ophiocordycipitaceae)

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**Academic editor:** Cecile Gueidan  |  Received 5 May 2022  |  Accepted 10 August 2022  |  Published 30 August 2022

**Citation:** Zou W, Tang D, Xu Z, Huang O, Wang Y, Tran N-L, Yu H (2022) Multigene phylogeny and morphology
reveal *Ophiocordyceps hydrangea* sp. nov. and *Ophiocordyceps bidoupensis* sp. nov. (Ophiocordycipitaceae). MycoKeys 92:
109–130. https://doi.org/10.3897/mycokeys.92.86160

**Abstract**

*Ophiocordyceps* species have a wide range of insect hosts, from solitary beetle larva to social insects. However,
among the species of *Ophiocordyceps*, only a few attack cicada nymphs. These species are mainly clustered
in the *Ophiocordyceps sobolifera* clade in *Ophiocordyceps*. A new entomopathogenic fungus parasitic on
cicada nymphs, and another fungus parasitic on the larva of Coleoptera, are described in this study. The
two new species viz. *Ophiocordyceps hydrangea* and *Ophiocordyceps bidoupensis* were introduced based on
morphology and multigene phylogenetic evidence. The phylogenetic framework of *Ophiocordyceps* was
re-constructed using a multigene (\(nrsu\), \(nrLSU\), \(tef-1\), \(rpb1\), and \(rpb2\)) dataset. The phylogenetic analyses
results showed that *O. hydrangea* and *O. bidoupensis* were statistically well-supported in the *O. sobolifera*
clade, forming two separate subclades from other species of *Ophiocordyceps*. The distinctiveness of these
two new species was strongly supported by both molecular phylogeny and morphology.

**Keywords**

2 new taxa, entomopathogenic fungi, morphology, phylogenetic analyses

* Those authors contributed equally to this work.

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Introduction

_Ophiocordyceps_ G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora is the largest genus in the Ophiocordycipitaceae, comprising approximately 290 species. It was originally established by Petch, with _Ophiocordyceps blattae_ Petch as the type species (Petch 1931). According to the arrangement of the perithecia, the size of asci, ascospores, and secondary ascospores, _Ophiocordyceps_ was transferred to _Cordyceps_ sensu lato by Kobayasi, as a subgenus of _Cordyceps_ s.l. (Kobayasi 1941, 1982). Sung et al. (2007) used five to seven loci combined molecular datasets to revise the _Cordyceps_ and the Clavicipitaceae. The species of _Cordyceps_ and Clavicipitaceae were divided into three families (Corycycipitaceae, Ophiocordycipitaceae, Clavicipitaceae sense stricto) and four genera (_Cordyceps_ sense stricto, _Ophiocordyceps_, _Elaphocordyceps_, and _Metacordyceps_). The research results of Sung et al. (2007) are currently the most widely accepted phylogenetic classification of _Cordyceps_ s.l. In 2015, _Ophiocordyceps_ was divided into _O. ravenelii_ clade, _O. unilateralis_ clade, _O. sobolifera_ clade, and _O. sphecocephala_ clade by Sanjuan et al. With the continuous revision of _Ophiocordyceps_, it has now been divided into four clades, including the _Hirsutella_ clade, _O. sobolifera_ clade, _O. sphecocephala_ clade, and _O. ravenelii_ clade (Mains 1958; Sung et al. 2007; Quandt et al. 2014; Sanjuan et al. 2015; Simmons et al. 2015; Wang et al. 2018). Many phylogenetic classifications for entomopathogenic fungi have been revised in recent studies (Wang et al. 2018; Fan et al. 2021; Wang et al. 2021a, 2021b).

There are fewer species in the _O. sobolifera_ clade than in the _Hirsutella_ clade and the _O. sphecocephala_ clade. The _O. sobolifera_ clade is statistically well-supported in most studies and 11 species have been described in the Index Fungorum (Kobayasi and Shimizu 1963; Hywel-Jones 1995b; Sung et al. 2007, 2011; Luangs-ard et al. 2008; Hyde et al. 2017; Crous et al. 2018, 2019; Lao et al. 2021; Wang et al. 2021a). Asexual morphs of _Ophiocordyceps_ were reported as _Hirsutella_ Pat., _Paraisaria_ Samson & B.L. Brady, _Sorosporella_ Sorokin, _Hymenostilbe_ Petch and _Syngliocladium_ Petch, etc. (Sung et al. 2007; Quandt et al. 2014). In most species of _Ophiocordyceps_, their dominant asexual morphs were _Hirsutella_, the conidiogenous cells basally swollen that taper to a narrow neck, producing a mucilaginous cluster of one or several conidia (Simmons et al. 2015; Wang et al. 2018).

_Ophiocordyceps_ species have a wide range of insect hosts, from solitary beetle larvae to social insects. More than 10 insect orders were attacked, including Hemiptera, Coleoptera, Lepidoptera, Blattaria, Dermaptera, Diptera, Hymenoptera, Isoptera, Megaloptera, and Mantodea (Araújo et al. 2015; Araújo and Hughes 2016, 2019). Entomopathogenic fungi whose hosts are cicada nymphs have attractive stromata. The most typical representative of this group was _Cordyceps cicadae_ (Miquel) Massee (Massee 1895) in Cordycipitaceae, with the stroma like a flower (Sung et al. 2007). However, for species of _Ophiocordyceps_, with cicada nymph hosts including _O. khonkaenensis_ Tasanathai, Thanakitpipattana & Luangsa-ard (Crous et al. 2019), _O. sobolifera_ (Hill ex Watson) G.H. Sung, J.M. Sung,
Hywel-Jones & Spatafora (Kobayasi and Shimizu 1963; Sung et al. 2007), and *O. longissima* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Kobayasi and Shimizu 1963; Sung et al. 2007, 2011) in *O. sobolifera* clade, their stromata were typically bright-colored and cylindrical. The hosts of the entomopathogenic fungi within the *O. sobolifera* clade were divided into two categories. One group with Hemiptera hosts was represented by *O. sobolifera*. These fungi had a hard texture stroma, which was cylindrical, and deep-colored, and had swollen fertile parts (Kobayasi and Shimizu 1963; Sung et al. 2011; Crous et al. 2019). Another group had Coleoptera hosts that were characterized by hard texture stromata, being cylindrical, bright-colored, and with a sterile apices cone at the top of the stroma (Hywel-Jones 1995b; Luangsa-ard et al. 2008; Crous et al. 2018; Lao et al. 2021; Wang et al. 2021a).

*Cordyceps* s.l. is globally distributed with the highest species diversity recorded in subtropical and tropical regions (Nguyen and Vo 2005; Ban et al. 2015; Doan et al. 2017; Luangsa-ard et al. 2018), especially in East and Southeast Asia (Sung et al. 2007; Fan et al. 2021; Wang et al. 2021a). To date, more than 800 species of *Cordyceps* and *Ophiocordyceps* have been named worldwide, and there are at least 200 species in China (Index Fungorum 2022). Yunnan Province, located in southwest China, has unique geographical and ecological features. Many species of *Ophiocordyceps* were reported from Yunnan, including *O. alboperitheciata* H. Yu, Q. Fan & Y.B. Wang (Fan et al. 2021), *O. furcatosubulata* H. Yu, Y. Wang & Y.B. Wang (Wang et al. 2021a), *O. highlandensis* Zhu L. Yang & J. Qin (Yang et al. 2015), *O. lanpingensis* H. Yu & Z.H. Chen (Chen et al. 2013), *O. laojunshanensis* J.Y. Chen, Y.Q. Cao & D.R. Yang (Chen et al. 2011), *O. liangshanensis* (M. Zang, D.Q. Liu & R.Y. Hu) H. Yu, Y. Wang, Y.D. Dai, Zhu L. Yang & Y.B. Wang (Wang et al. 2021b), and *O. pingbianensis* H. Yu, S.Q. Chen & Y.B. Wang (Chen et al. 2021). The unique geographical conditions of Yunnan have resulted in high *Cordyceps* s.l. species diversity. There is also a high species diversity of *Cordyceps* s.l. in Southeast Asia, where more than 500 species of entomopathogenic fungi have been reported. Approximately 400 species of entomopathogenic fungi are distributed in Thailand (Sung et al. 2007; Luangsa-ard et al. 2011, 2018; Ban et al. 2015; Tasanathai et al. 2019; Xiao et al. 2019). Vietnam is second to Thailand, in the number of entomopathogenic fungi species, with more than 100 species having been reported such as *Moelleriella pumatensis* T.T. Nguyen & N.L. Tran (Mongkolsamrit et al. 2011), *O. furcatosubulata* H. Yu, Y. Wang & Y.B. Wang (Wang et al. 2021a), and *O. puluongensis* H. Yu, Z.H. Xu, N.L. Tran & Y.B. Wang (Xu et al. 2022). These findings suggested that Vietnam should be abundant in species diversity of *Cordyceps* s.l. (Mongkolsamrit et al. 2011; Doan et al. 2017; Luyen et al. 2017).

Several studies have evaluated the taxonomy and biology of entomopathogenic fungi, especially species found in China and Southeast Asia. In this study, one unknown species of *Ophiocordyceps* attacking a cicada nymph was collected from Yunnan Province, Jinghong City, Nabanhe National Nature Reserve, in China. Another...
Table 1. Specimen information and GenBank accession numbers of the sequences used in this study.

| Species                      | Host                        | Isolate no./specimen no. | GenBank accession no. |
|------------------------------|-----------------------------|--------------------------|-----------------------|
|                              |                             |                          | nrSSU                 |
| Hirsutella citriformis       | Cixiidae (Hemiptera)        | ARSEF 1446               | KM652065              |
| Hirsutella fusiformis        | Brevicoryne incarnata       | ARSEF 5474               | KM652067              |
| Hirsutella gigantea          | Pammulilidae (Hymenoptera) | ARSEF 30                 | --                    |
| Hirsutella guajana           | Empoasca kueneri           | ARSEF 878                | KM652068              |
| Hirsutella illatissir        | Erisissa langenseri         | ARSEF 5539               | KM652069              |
| Hirsutella kirchneri         | Allocaeus hystric           | ARSEF 5551               | KM652070              |
| Hirsutella lecaniicola       | Parthenolecanium corni      | ARSEF 8888               | KM652071              |
| Hirsutella liboensis         | Larva of Cossidae (Lepidoptera) | ARSEF 9603            | KM652072              |
| Hirsutella necatrix          | Acari                       | ARSEF 5549               | KM652073              |
| Hirsutella nodulata          | Dioryctria zimmermani      | ARSEF 5473               | KM652074              |
| Hirsutella radiata           | Diptera                     | ARSEF 1369               | KM652076              |
| Hirsutella rhosilensis       | Menocerisoma xenuplae (Cocciidae, Hymenoptera) | ARSEF 3747          | KM652080              |
| Hirsutella strigosa          | Nephotettix virescens       | ARSEF 2197               | KM652085              |
| Hirsutella subulata          | Microlepidoptra (Lepidoptera) | ARSEF 2227            | KM652086              |
| Hirsutella thompsonii var.  | Acrisia seldenii            | ARSEF 2459               | KM652099              |
| Hirsutella thompsonii var.  | Pheriloptera alevrain       | ARSEF 137                | KM652087              |
| Hirsutella thompsonii var.  | Acalauta succincta          | ARSEF 254                | KM652101              |
| Ophiocordyceps acicularis   | Larva of Coleoptera         | ARSEF 110987             | EF468950              |
| Ophiocordyceps acicularis   | Larva of Coleoptera         | ARSEF 110988             | EF468951              |
| Ophiocordyceps agristidii    | Larva of Coleoptera         | ARSEF 5602               | DQ522540              |
| Ophiocordyceps annulata     | Larva of Coleoptera         | CEM 303                  | KJ878819              |
| Ophiocordyceps aphidii      | Larva of Scarabaeidae       | ARSEF 548                | DQ522541              |
| Ophiocordyceps appendiculata| Larva of Coleoptera         | NBRC 108060              | JN941728              |
| Ophiocordyceps arborescens  | Larva of Pueraria lobata (Lepidoptera) | NBRC 105891           | AB968386              |
| Ophiocordyceps bidoupensis  | Larva of Elateridae (Coleoptera) | YFCC 8793            | OM304638              |
| Ophiocordyceps brunnatentata| Larva of Coleoptera         | BDRC 18211               | EF468952              |
| Ophiocordyceps brunnei     | Hemiptera                  | TNS F1853                | KJ878933              |
| Ophiocordyceps cochlioides  | Cochlioididae papa (Lepidoptera) | HMAS 199612          | KJ878917              |
| Ophiocordyceps cruciferum   | Larva of Elateridae (Coleoptera) | OSC 128576           | DQ522542              |
| Ophiocordyceps crevicornis  | Coccinellidae (Hemiptera) | TBRC 8095                | --                    |
| Ophiocordyceps cinnabarinus| Larva of Coleoptera         | TBRC 8100                | --                    |
| Ophiocordyceps cruciferum   | Hemiptera                  | TNS F1857                | --                    |
| Ophiocordyceps cruciferum   | Hemiptera                  | TNS F1857                | --                    |
| Ophiocordyceps formicarum   | Larva of Coleoptera         | TBRC 8095                | --                    |
| Ophiocordyceps furciferum   | Larva of Coleoptera         | TBRC 8100                | --                    |
| Species                          | Host                                      | GenBank accession no. | mLSU        | nrLSU        | nrS        | Su        | tef-α     | rpB1      | rpB2      |
|---------------------------------|-------------------------------------------|-----------------------|-------------|--------------|------------|-----------|-----------|-----------|-----------|
| Ophiocordyceps forquignonii    | Adult fly (Diptera)                        | KJ878912 – KJ878991  | KJ878876    | –            | –          | –         | –         | –         | –         |
| Ophiocordyceps furcatosubulata | Larva of Elateridae (Coleoptera)           | MT774216 – MT774240  | MT774223    | MT774244     | MT774230   | MT774237  | –         | –         | –         |
| Ophiocordyceps furcatosubulata | Larva of Elateridae (Coleoptera)           | YHH 17005             | –           | –            | –          | –         | –         | –         | –         |
| Ophiocordyceps geometricioida  | Larva of Geometridae (Lepidoptera)         | TBRC 8095             | –           | MF614648     | MF614632   | MF614663  | MF614679  | –         | –         |
| Ophiocordyceps houaynhangensis | Larva of Coleoptera                        | TBRC 8428             | –           | MH928992     | –          | –         | –         | –         | –         |
| Ophiocordyceps hydrangea        | Nymph of cicada (Hemiptera)                | YFCC 8832             | –           | –            | –          | –         | –         | –         | –         |
| Ophiocordyceps hydrangea        | Nymph of cicada (Hemiptera)                | YFCC 8833             | –           | –            | –          | –         | –         | –         | –         |
| Ophiocordyceps hydrangea        | Nymph of cicada (Hemiptera)                | YFCC 8834             | –           | –            | –          | –         | –         | –         | –         |
| Ophiocordyceps karstii          | Heptadia jianchuansis (Lepidoptera)        | MFLU:15-3884          | KJ854952    | –            | –          | KJ854945  | KJ854943  | –         | –         |
| Ophiocordyceps kimflemingiae    | Camponotus castaneus (Hymenoptera)         | SC09B                 | KX713631    | KX713620     | KX713724   | –         | –         | –         | –         |
| Ophiocordyceps knipholosioides  | Gephalotes attatus adult ant (Hymenoptera)| HUA 168614            | KG610790    | KG658679     | KG610793   | KG658667  | KG610717  | –         | –         |
| Ophiocordyceps houaynhangensis | Larva of Coleoptera                        | DL 0817               | MT928355    | MT928306     | –          | –         | –         | –         | –         |
| Ophiocordyceps longissima       | Cicada nymph (Cicadidae, Hemiptera)        | NBRC 100695           | AB968392    | AB968420     | AB968584   | –         | AB968546  | –         | –         |
| Ophiocordyceps macrocarunculata | Larva of Cossidae (Lepidoptera)            | NBRC 100685           | AB968388    | AB968416     | AB968574   | –         | AB968536  | –         | –         |
| Ophiocordyceps multiperitheciata| Lepidoptera larva                          | BCC 69008             | MF614657    | MF614641     | MF614682   | –         | MF614678  | –         | –         |
| Ophiocordyceps myrmicarum       | Hymenoptera (Formicidae)                   | HIRS 45               | KJ80150     | JX560665     | JX560973   | KJ80151   | –         | –         | –         |
| Ophiocordyceps nigella          | Larva of Lepidoptera                       | EFCC 9247             | EF68963     | EF68818      | EF68758    | EF68866   | EF68920   | –         | –         |
| Ophiocordyceps pruinosa         | Hemiptera                                 | NHJ 12994             | EU360916    | EU360924     | EU360963   | EU360984  | –         | –         | –         |
| Ophiocordyceps pseudobaccalarii | Larva of Lepidoptera                       | TBRC 8102             | MF614646    | MF614630     | MF614661   | MF614677  | –         | –         | –         |
| Ophiocordyceps pulvinata        | Camponotus adult ant                       | TNS-F 30044           | GU930428    | AB721305     | GU930429   | GU930420  | –         | –         | –         |
| Ophiocordyceps ramosissimum     | Phasus nodus larva                         | GZUHNN8               | KJ028012    | KJ028014     | KJ028017   | –         | –         | –         | –         |
| Ophiocordyceps ravenelli       | Beetle larva (Coleoptera)                  | OSC 110995            | DQ522550    | DQ518764     | DQ522334   | DQ522379  | DQ522430  | –         | –         |
| Ophiocordyceps robertsi        | Larva of Heptidae (Lepidoptera)            | KEW 27083             | EF68826     | EF688766     | –          | –         | –         | –         | –         |
| Ophiocordyceps rubiginosiperitheciata | Larva of Coleoptera                  | NBRC 100696           | JN941704    | JN941437     | AB968582   | JN992348  | AB968544  | –         | –         |
| Ophiocordyceps spataforae      | Hemiptera (nymph)                          | NBRC 100697           | AB968395    | AB968422     | AB968590   | –         | –         | –         | –         |
| Ophiocordyceps sphecocephala   | Larva of Hymenoptera (Lepidoptera)         | TNS-F18521            | KJ878933    | KJ878989     | KJ87979    | KJ87903   | –         | –         | –         |
| Ophiocordyceps sobolifera      | Cicada nymph (Cicadidae, Hemiptera)        | TNS F18521            | KJ878933    | KJ878989     | KJ87979    | KJ87903   | –         | –         | –         |
| Ophiocordyceps sobolifera      | Hemiptera (nymph)                          | NBRC 100697           | AB968395    | AB968422     | AB968590   | –         | –         | –         | –         |
| Ophiocordyceps spataforae      | Hemiptera adult                            | NHJ 12525             | EF690125    | EF690078     | EF69063    | EF69092    | EF69111   | –         | –         |
| Ophiocordyceps spataforae      | Hemiptera adult wap                        | NBRC 101753           | JN941695    | JN941446     | AB968592   | JN992429  | AB968553  | –         | –         |
| Ophiocordyceps stylophora      | Larva of Elateridae (Coleoptera)           | OSC 110999            | EF690832    | EF688777     | EF68882    | EF68931   | –         | –         | –         |
| Ophiocordyceps thanathonensis  | Hymenoptera adult                          | MFLU 16-2910          | MF882926    | MF850377     | MF872614   | MF872616  | –         | –         | –         |
unknown species of *Ophiocordyceps* attacking larvae of Elateridae was collected from Lintong Province, Bidoup Nuiba National Park, in Vietnam. The phylogeny and morphology of these two fungi were determined, and their systematic position was established in Ophiocordycipitaceae. The phylogenetic analyses results showed that the two new species belonged to *Ophiocordyceps*, and were named *Ophiocordyceps hydrangea* and *Ophiocordyceps bidoupensis* based on well-supported morphology and molecular data.

**Materials and methods**

**Sample collection and isolation**

The specimens were collected from China and Vietnam, and the collection site information was noted, including altitude, longitude, latitude, and habitat type. Samples were placed in sterilized tubes or plastic bags and boxes, returned to the laboratory, and stored at 4 °C. The specimens were photographed using a Canon 750D camera (Canon Inc., Tokyo, Japan). The size was measured, and characteristics were recorded including length of the stroma, single or multiple, length and width of stipe clavate and fertile parts, shape, texture, and color. To obtain axenic cultures, the segments were removed from insect bodies, and these segments were placed onto Potato Dextrose Agar (PDA) consisting of peptone and yeast powder (potato 100 g/500 mL, dextrose 10 g/500 mL, agar 10 g/500 mL, yeast powder 5 g/500 mL, peptone 2.5 g/500 mL) plates. The plates were placed in a culture room at 25 °C until isolated into pure cultures. The cultures were saved on a PDA slant (to grow slowly), and stored at 4 °C. All specimens were deposited in the Yunnan Herbal Herbarium (YHH) of Yunnan University. The extypes of the two species were deposited in the Yunnan Fungal Culture Collection (YFCC) of Yunnan University.
Morphological observations

To describe the sexual morphs of the two species, frozen sections or hand sections of the fruiting structures of the stroma were immersed in water and then dyed with lactophenol cotton blue solution for morphological observation and photomicrography (Wang et al. 2021a). For observations on asexual morphs, new colonies were established from old cultures and placed on new PDA plates. The plates were cultured in an incubator for 6 or 12 weeks at 25 °C, and then asexual morphs were observed and recorded (shape, texture, and color of the colonies). Microscope slide cultures were made using the methods of Wang et al. (2020). The morphological observations and measurements were made using Olympus CX40 and BX53 microscopes.

DNA extraction, PCR, and sequencing

Five-centimeter segments from the stroma of fresh specimens and the cultures were used for DNA extraction to ensure the cultures and specimens were the same. Total DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) according to the procedure described by Liu et al. (2001). The DNA was used for PCR amplification. The primer pair, NS4 (5’-CTTCCGTCAATTCTTTAAG-3’) and NS1 (5’-GTAGTCATATGCTTGTCTC-3’) was used to amplify nrSSU (the nuclear ribosomal small subunit) (White et al. 1990). The primer pair, LR5 (5’-ATCCTGAGG-GAAACTTC-3’) and LR0R (5’-GTACCCGCTGAACCTTAAGC-3’) was used to amplify nrLSU (the nuclear ribosomal large subunit) (Vilgalys and Hester 1990; Rehner and Samuels 1994). The primer pair, 983F (5’-GCYCYGGHCAYCGTGAY -TTYAT-3’) and 2218R (5’-ATGACACCRACRGRARGTYYTG-3’) was used to amplify tef-1α (the translation elongation factor 1α) (Rehner and Buckley 2005). The primer pair, CRPB1A (5’-CAYCCWGGYTTYATCAAGAA-3’) and RPB1C (5’-CCNGCDATNTCRTTTRCTTAATRA-3’) were used to amplify rpb1 (the largest subunit of RNA polymerase II) (Castlebury et al. 2004; Bischoff et al. 2006). The primer pair, fRPB2-5F (5’-GAYGAYMGWGATCAYTTYGG-3’) and fRPB2-7cR (5’-CCC-ATRGCTTGYTRYTCCCAT-3’) was used to amplify rpb2 (the second largest subunit of RNA polymerase II) (Liu et al. 1999). The polymerase chain reaction (PCR) for amplification of the five genes and their sequencing were described by Wang et al. (2015).

Phylogenetic analyses

Sequences of the five genes (nrSSU, nrLSU, tef-1α, rpb1, and rpb2) were downloaded from GenBank, and combined with the newly generated sequences in this study. The taxa information of the species and GenBank accession numbers of the five genes are listed in Table1. Sequences of the five genes were aligned using the Clustal X (v.2.0) and MEGA6 (v.6.0) (Larkin et al. 2007; Tamura et al. 2013). Ambiguously aligned sites were eliminated, and the gaps were treated as missing data. The aligned sequences of the five genes (nrSSU, nrLSU, tef-1α, rpb1, and rpb2) were concatenated into a single
combined dataset using MEGA6 (v.6.0.). Conflicts between the five genes were tested using PAUP* (v.4.0b10) (Swofford 2002). The results of the phylogenetic signals in the five genes were not in conflict. The concatenated dataset containing all five genes consisted of 11 data partitions, including one each for nrSSU and nrLSU, and three for each of the three codon positions of tef-1α, rpb1, and rpb2. Phylogenetic analyses based on the five genes were made using BI and ML methods (Ronquist and Huelsenbeck 2003; Stamatakis et al. 2008). We used the optimal model GTR+I with 1,000 rapid bootstrap replicates on the five genes for ML analyses (Stamatakis 2006). We conducted BI analyses using a GTR+G+I model determined by jModelTest (v.2.1.4), conducted on MrBayes (v.3.1.2) for 5 million generations (Darriba et al. 2012). The phylogenetic tree constructed was viewed and edited using FigTree (v.1.4.2) and Adobe Illustrator CS6.

Results

Phylogenetic analyses

A total of 83 samples were used for the phylogenetic analyses. Five gene sequences of the two new species collected were used to reconstruct the phylogenetic framework of Ophiocordyceps. Two taxa of Tolypocladium were designated as the outgroup, and these were, respectively, Tolypocladium ophioglossoides CBS 100239 and Tolypocladium inflatum OSC 71235. The alignment lengths of the 83 samples were composed of 4,486 bp sequence data, 971 bp of nrSSU, 921 bp of nrLSU, 943 bp of tef-1α, 726 bp of rpb1, and 925 of rpb2. The phylogenetic tree showed that these were identical in overall topologies to previous studies. Four clades (Hirsutella clade, O. sobolifera clade, O. sphecocephala clade, and O. ravenelii clade) of Ophiocordyceps were well-supported by ML bootstrap proportions and BI posterior probabilities (Fig. 1). The two new species in the O. sobolifera clade, O. hydrangea and O. bidoupensis, formed two separate subclades. Three samples of O. hydrangea (BP = 98%, PP = 1) formed a separate subclade with O. longissima and O. yakusimensis, while O. bidoupensis (BP = 83%, PP = 0.99) formed a separate subclade with O. houaynhangensis.

Taxonomy

Ophiocordyceps hydrangea H. Yu, W.Q. Zou & D.X. Tang, sp. nov.
MycoBank No: 843203
Fig. 2

Etymology. Hydrangea, referred to the top of the stroma similar to hydrangea.

Holotype. CHINA, Yunnan Province, Jinghong City, Nabanhe National Nature Reserve, 22°8'21.32"N, 100°42'18.35"E, alt. 612 m, on cicada nymphs (Cicadidae, Hemiptera). The material was found in the soil of an evergreen broad-leaved forest, 18 August 2020, H. Yu (YHH 20081, holotype; YFCC 8834, ex-holotype culture).
Multigene phylogeny and morphology of two new species

**Figure 1.** Phylogenetic relationships of *Ophiocordyceps hydrangea* and related species from the five genes dataset (nrLSU, nrSSU, tef-1α, rpb1, and rpb2) based on ML and BI analyses. Statistical support values of BI posterior probabilities and ML bootstrap proportions (0.5/≥50%) are shown at the nodes.
Sexual morph. The stroma was grown from the head of the host cicada nymph, solitary, the top of the stroma similar to hydrangea, pale pink, 1.6–6.4 cm long. Sexual morph was not observed.

Asexual morph. The colony grew slowly on PDA medium. Cultured at 25 °C for about 12 weeks, the diameter of the colony was 25–28 mm, pale pink, the edge white, hard texture. The back of the colony was white to brown. Surface hyphae rough,

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Figure 2. Ophiocordyceps hydrangea A, B fungus on a cicada nymph C, D colony on PDA medium E conidiophores, conidiogenous cells and conidia F–J conidiogenous cells and conidia K conidia. Scale bars: 1 cm (A, B); 2 cm (C, D); 10 μm (E, F, G, I, J); 5 μm (H, K).
hyaline, septate. Conidiophores were cylindrical. Conidiogenous cells were solitary or whorled, ampuliform, smooth-walled, forming on conidiophores or colonies, hyaline, with swollen base, and slender top, 10.6–17.6 µm long, 2.9–4.3 µm wide at the swollen base, and 1.1–2.2 µm wide at the slender top. Conidia hyaline, ovoid or long oval, solitary, 6.8–10.1 × 3.3–4.5 µm.

**Host.** Cicada nymph (Cicadidae, Hemiptera).

**Habitat.** In the soil of an evergreen broad-leaved forest.

**Distribution.** China.

**Other material examined.** China, Yunnan Province, Jinghong City, Nabanhe National Nature Reserve, 22°8′21.32″N, 100°42′18.35″E, alt. 612 m, on cicada nymphs (Cicadidae, Hemiptera) was found in the soil an evergreen broad-leaved forest, 18 August 2020, H. Yu (YFCC 8832, YFCC 8833).

**Notes.** Phylogenetic analyses showed that *O. hydrangea* clustered with *O. sobolifera*, *O. longissima*, and *O. yakusimensis* of the *O. sobolifera* clade (Fig. 1). Their hosts were cicada nymphs compared to other species of the *O. sobolifera* clade (Table 2). *Ophiocordyceps hydrangea* was well supported by BI and ML results, forming a separate subclade with *O. sobolifera*, *O. longissima*, and *O. yakusimensis*. The macro-morphology of *O. hydrangea* was clearly different from *O. sobolifera*, *O. longissima*, *O. khonkaenensis*, and *O. yakusimensis*. The stroma of *O. hydrangea* grew from the head of the host cicada nymph, solitary, and the top of the stroma was like a pale pink hydrangea.

**Ophiocordyceps bidoupensis** H. Yu, W.Q. Zou & D.X. Tang, sp. nov.
MycoBank No: 843204

**Etymology.** Bidoupensis, referred to the type species collected from Bidoup Nuiba National Park.

**Holotype.** VIETNAM, Lintong Province, Bidoup Nuiba National Park, 12°8′9.30″N, 108°31′51.38″E, alt. 1678 m, on larva of Elateridae (Coleoptera) buried in soil, emerging from the leaf litter on the forest floor, 16 October 2017, H. Yu (YHH 20036, holotype; YFCC 8793, ex-holotype culture).

**Sexual morph.** The stroma grew from the head of the host, solitary, solid, cylindrical, 11.8–22.5 cm long, yellow. Stipe clavate, yellow, curved, 10.7–21.2 cm long, 0.7–0.9 mm wide. Fertile parts cylindrical, yellow, slightly curved, 2.9–11.3 mm long, 0.9–1.6 mm wide. Sterile apices cone, yellow, 2.1–7.2 mm long, 0.2–0.7 mm wide. Perithecia immersed, pyriform to lanceolate, brown-yellow, 213.4–405.9 × 74.8–192.4 µm. Asci hyaline, slender, 116.1–192.7 × 4.8–7.5 µm. Asci cap prominent, capitulate, 4.7–6.1 × 3.3–5.4 µm. Ascospores hyaline, filiform, multi-septate.

**Asexual morph.** The colony grew slowly on PDA medium. Cultured at 25 °C for about 6 weeks, the diameter of the colony was 38–45 mm, white, aerial mycelium on the surface, slightly convex. The back of the colony was grayish-white, dark brown in the middle. Surface smooth of hyphae, hyaline, septate. Conidiogenous cells cone, hyaline, septate, smooth-walled, forming on hyphae, with a hypertrophic base,
tapering abruptly to a thin neck, 13.80–46.4 × 0.42–5.13 µm. Conidia hyaline, oval or briolette, smooth-walled, 2.24–3.61 × 1.49–2.70 µm.

**Host.** Larva of Elateridae (Coleoptera).

**Habitat.** The hosts were buried in soil, and the stroma were found in the leaf litter on the forest floor.

**Distribution.** Vietnam.

Figure 3. *Ophiocordyceps bidoupensis* A–C fungus on an Elateridae larva D, E cross-section of the ascoma showing the perithecial arrangement F–H asci I ascospores J, K colony on PDA medium L–N conidiogenous cells and conidia O conidiogenous cells P, Q conidia. Scale bars: 1 cm (A–C); 200 µm (D); 20 µm (E–H); 10 µm (I); 2 cm (J, K); 5 µm (L–Q).
Notes. Phylogenetic analyses showed that *O. bidoupensis* was clustered with *O. houaynhangensis*, *O. brunneipunctata*, *O. langbianensis*, *O. cossidarum*, and *O. furcatosubulata* of the *O. sobolifera* clade (Fig. 1). Their hosts were larvae of Elateridae compared to cicada nymph hosts of the other species of the *O. sobolifera* clade (Table 2). *Ophiocordyceps bidoupensis* was well-supported by bootstrap support and posterior probabilities, and formed a separate subclade with *O. houaynhangensis*, *O. brunneipunctata*, *O. langbianensis*, and *O. cossidarum*. The morphology of *O. bidoupensis* was clearly different in shape and size from other species of *O. sobolifera* clade (Table 2). The stroma of *O. bidoupensis* grew solitary from the head of the host; sterile apices of the stroma were different from the other species.

Discussion

*Ophiocordyceps* is the largest genus in the Ophiocordycipitaceae, with a wide range of hosts and various species. At present, more than 290 species of *Ophiocordyceps* have been reported (Index Fungorum 2022). However, only 11 species are described in the *O. sobolifera* clade and their hosts are mainly Coleoptera larvae and cicada nymphs (Hemiptera) (Table 2). We describe the new species *O. hydrangea* attacking cicada nymphs and the new species *O. bidoupensis* attacking Coleoptera larvae. Most species have diverse macro-morphological or micro-morphological characteristics due to the same entomopathogenic fungi having a different host, or different species of entomopathogenic fungi having the same host (Sung et al. 2007, 2011; Araújo et al. 2015; Araújo and Hughes 2016; Shrestha et al. 2016; Luangsa-ard et al. 2018; Crous et al. 2019; Fan et al. 2021; Wang et al. 2021a). Hemiptera hosts are widely present among the species of *Ophiocordyceps*, including species of the *Hirsutella* clade, *O. sobolifera* clade, *O. sphaecocephala* clade, and *O. raveneli* clade.

The entomopathogenic fungi whose host is Hemiptera have diverse morphological characteristics. For example, *O. mutans* (Patouillard) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Sung et al. 2007), its hosts were stink bugs (Hemiptera), stromata solitary or multiple, fertile parts was red (Hywel-Jones 1995a; Luangsa-ard et al. 2008), stromata of *O. brunneinigra* (Hemipteran host) were flexuous, arising from between the head and the thorax of the host (Luangsa-ard et al. 2018), stromata of *O. spataforae* Tasanathai, Thanakipipattana, Khonsanit & Luangsa-ard were cylindrical, cream to pale brown (Luangsa-ard et al. 2018). However, from previously reported Hemipteran hosts, only a few hosts of the *O. sobolifera* clade were cicada nymphs in *Ophiocordyceps* (Kobayasi and Shimizu 1963; Sung et al. 2011; Crous et al. 2019). In this study, the host of *O. hydrangea* was a cicada nymph. More interestingly, the *O. hydrangea* was significantly more beautiful than other species; the stroma grew from the head of the host cicada nymph, and the top of the stroma like a hydrangea (Sung et al 2007, 2011; Crous et al. 2019). Coleoptera hosts were common in species of *Ophiocordyceps*. More than 20 species of *Ophiocordyceps* were parasitic on Coleoptera larvae (Shrestha et al. 2016). These species included *O. acicularis* (Ravenel) Petch (Petch 1933), *O. annulata* (Kobayasi & Shimizu) Spatafora, Kepler & C.A. Quandt (Kobayasi and Shimizu 1982; Spatafora et al. 2015), *O. aphodii*
| Species              | Host                        | Stromata                                      | Perithecia                                      | Asci                                      | Ascospores                                      | Conidiogenous cells                                   | Conidia                                      | References                                      |
|----------------------|-----------------------------|-----------------------------------------------|------------------------------------------------|------------------------------------------|------------------------------------------------|------------------------------------------------------|-----------------------------------------------|------------------------------------------------|
| O. bidoupensis       | Larva of Elateridae (Coleoptera) | Solitary, solid, cylindrical, yellow, 11.8–22.5 cm long. | Immersed, pyriform to lanceolate, brown-yellow, 213.4–405.9 × 74.8–192.4 μm. | Hyaline, slender, 116.1–192.7 × 4.8–7.5 μm. | Hyaline, filiform, multi-septate. | Cone, hyaline, septate, smooth-walled, forming on hyphae, with a hypertrophic base, tapering abruptly into a thin neck, smooth-walled, 13.8–46.4 × 0.42–5.13 μm. | Oval or briolette, hyaline, smooth-walled, 2.24–3.61 × 1.49–2.70 μm. | This study                                      |
| O. brunneipunctata   | Larva of Elateridae (Coleoptera) | Solitary, rarely up to 3, simple, 25–90 mm high. | Immersed, perithecioid, brown, ovate to pyriform, brown-walled, 355–454 × 136–171 μm. | Hyaline, cylindrical, 8-spored, 174–221 × 5.7–7 μm. | Hyaline, filiform, multi-septate; 131–153 × 1.8–2.2 μm, breaking into 32 part-spores. | – | – | Hywel-Jones 1995b; Luangsawad 2008 |
| O. cossidarum        | Larva of Cossidae (Lepidoptera) | Solitary, simple, 40–70 mm high. | Immersed, red, ovate to phialide, red-walled, 8–12 μm. | Hyaline, cylindrical, 138.8–202.5 × 4.3–6.0 μm. | Hyaline, filiform, multi-septate, finally breaking into secondary ascospores, 3.7–5.3 × 1.3–2.0 μm. | Polyphialidic, forming on conidiophores or side branches, hyaline, with a slender or subulate base, tapering gradually, smooth-walled or verruculose, 3.5–15.8 × 0.9–1.7 μm. | Solitary, asperate, smooth-walled, broadly ellipsoid or ellipsoid, 1.5–2.5 × 1.2–1.9 μm. | Wang et al. 2021a |
| O. ferratombulata     | Larva of Elateridae (Coleoptera) | Single, solid, yellow to brown, 40–80 mm long, 1.5–2.2 mm wide. | Immersed, long ovoid or pyriform, 289.6–405.8 × 87.0–159.2 μm. | Hyaline, cylindrical, 200–250 × 5.0–6.0 μm. | Hyaline, filiform, multi-septate, finally breaking into secondary ascospores, 5.7–5.5 × 1.3–2.0 μm. | Monophialidic, philadés shaped with long necks, up to 30 μm long and 2–4 μm in breadth; philadés necks up to 18 μm long and 0.5 μm in breadth. | Hyaline, smooth, spherical, 2–3 μm. | Crous et al. 2018 |
| O. houaynhangensis   | Larva of Coleoptera          | Solitary, cylindrical, cream, up to 11 cm long and 1.5–2.5 mm in width. | Completely immersed, obclavate, 300–450 × 80–170 μm. | Cylindrical, 100–250 × 4.7–5.5 μm. | Hyaline, cylindrical, breaking into 32 small truncate part-spores, 4–7 × 1–2 μm. | Monophialidic, phiadés flattened-shaped with long necks, up to 30 μm long and 2–4 μm in breadth; phiadés necks up to 18 μm long and 0.5 μm in breadth. | Hyaline, smooth, spherical, 2–3 μm. | Crous et al. 2018 |
| O. langbianensis      | Larva of Coleoptera          | Solitary, rarely branched, 40–100 mm long. | Immersed, ovate or pyriform, 260–400 × 100–190 μm. | Cylindrical, with thickened cap, 200–250 × 5.0–6.0 μm. | Filiform, multi-septate, articulated in long-chain after discharging, sometimes breaking into 1-celled part-spores, 5–7.5 × 1.3–2 μm. | Divergent. | Chains, elliptical. | Lao et al. 2021 |
| O. sobolifera         | Cicada nymph (Cicadidae, Hemiptera) | Commonly single, rarely fasciculated by twos or threes, arising from head among polster, clavate or cylindrical 2–8 cm long, 2–6 mm thick, become hollow after maturity. | Rectangularly immersed, ampullaceous 500–600 × 220–260 μm, with somewhat long neck, ostiole somewhat prominent, walls hyaline 8–16 μm thick. | Cylindrical, 400–470 × 5.6–6.3 μm. | Finally breaking into secondary ascospores, truncate at both ends, 6–12 × 1.0–1.3 μm. | – | – | Kobayasi and Shimizu 1963 |
| O. subaerimensis      | Cicada nymph (Cicadidae, Hemiptera) | Very long attaining 14 cm, arising from the apical part between eyes. | Wholly embedded, narrow ovoid or almost naviculate, 740–800 × 170–230 μm, without protruding ostiole, neck almost destitute, wall 21–23 μm thick, composed of very thin cells. | Cylindrical, 270–310 × 5 μm. | Finally breaking into secondary ascospores, long cylindrical, somewhat attenuated on both sides, terminally truncate, 10–15 × 1 μm. | – | – | Kobayasi and Shimizu 1963 |
| Species            | Host                      | stromata                        | Perithecia | Asci                  | Ascospores            | Conidiogenous cells | Conidia               | References       |
|--------------------|---------------------------|---------------------------------|------------|-----------------------|-----------------------|---------------------|-----------------------|-------------------|
| *O. longissima*    | Cicada nymph (Cicadidae, Homoptera) | 5–20 cm long, sometimes much longer. | Ovoid to long ovoid, with a short neck, 440–590 × 130–300 μm. | 190–350 × 5–6 μm. | –                     | –                   | –                     | Sung et al. 2011 |
| *O. khonkaenensis* | Cicada nymph (Hemiptera)  | Variable in number, solitary to three, 20–30 mm long and 2–3 mm in breadth. | Immersed, flask shaped, 590–700 × 200–300 μm. | Cylindrical, 237.5–337.5 × 5–6 μm. | Filliform, 300–360 × 1–1.5 μm, readily breaking into 32 parts, 7–13 × 1–1.5 μm. | Phialidic, hirsutella-like, 5.5–11 × 2–3 μm. | Hyaline, fusiform, smooth-walled, 3–5.5 × 1–3 μm. | Crous et al. 2019 |
| *O. hydrangea*     | Cicada nymph (Cicadidae, Hemiptera) | Solitary, the top of the stroma similar to hydrangea, pale pink, 1.6–6.4 cm long. | – | – | – | – | – | This study |

Multigene phylogeny and morphology of two new species.
G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Mathieson 1949; Sung et al. 2007), *O. brunneipunctata* (Hywel-Jones) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Hywel-Jones 1995b; Sung et al. 2007; Luangsa-ard et al. 2008), *O. furcatusubulata* H. Yu, Y. Wang & Y.B. Wang (Wang et al. 2021a), *O. houaynhangensis* Keochanpheng, Thanakitp., Mongkol., & Luangsa-ard (Crous et al. 2018), *O. langbianensis* T.D. Lao, T.A.H. Le & N.B. Truong (Lao et al. 2021), *O. melolonthae* (Tulasne & C. Tulasne) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Sung et al. 2007), and *O. ravenelii* (Berkeley & M.A. Curtis) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Sung et al. 2007). Most species with Coleopteran host occur in soil and have solid, cylindrical, and yellow stromata. This is consistent with the results of this study.

Phylogenetic analyses based on the data from five genes showed that our phylogenetic framework of *Ophiocordyceps* was consistent with previous studies (Sung et al. 2007, 2011; Quandt et al. 2014; Simmons et al. 2015; Crous et al. 2018, 2019; Wang et al. 2018, 2021a; Lao et al. 2021). The genus of *Ophiocordyceps* consists of four clades, including the *Hirsutella* clade, *O. sobolifera* clade, *O. sphecocephala* clade, and *O. ravenelii* clade. Phylogenetic analyses showed that *O. hydrangea* clustered with *O. sobolifera, O. longissima,* and *O. yakusimensis* in the *O. sobolifera* clade, and *O. bidoupensis* clustered with *O. houaynhangensis, O. brunneipunctata, O. langbianensis, O. cossidarum,* and *O. furcatusubulata* in the same clade. Species within the *O. sobolifera* clade had different hosts, and morphological characteristics. These two new species clustered in two separate subclades within the *O. sobolifera* clade. The hosts of one subclade were cicada nymphs with stromata cylindrical or sarciniform, bright-colored, conidia were macro (Kobayasi and Shimizu 1963; Crous et al. 2019), and the hosts of another subclade were Coleoptera, with stromata cylindrical, conidia small, and a sterile apex on top of the stroma (Hywel-Jones 1995b; Luangsa-ard et al. 2008; Crous et al. 2018; Lao et al. 2021; Wang et al. 2021a). Therefore, the species of the *O. sobolifera* clade could be divided into two separate subclades when more materials were collected.

The species of *O. sobolifera* clade had diverse morphological characteristics (Table 2). The entomopathogenic fungi with cicada nymph hosts shared similar characteristics, stromata solitary or multiple, cylindrical, and bright-colored. However, they also differed in morphology. For example, *O. sobolifera* lacked a protruding ostiole with immersed perithecia (Kobayasi and Shimizu 1963), and this seems to be contrary to *O. yakusimensis* (Kobayasi and Shimizu 1963). Stromata of *O. longissima* were longer than other species, and had a short neck in perithecia (Sung et al. 2011). Compared to the ovoid perithecia of *O. longissima* and *O. yakusimensis, O. khonkaenensis* was flask-shaped (Crous et al. 2019). The top of the stroma of *O. hydrangea* was similar to hydrangea, the size and shape of conidiogenous cells and conidia were different from *O. khonkaenensis* (Table 2). The entomopathogenic fungi using Coleoptera hosts shared similar characteristics, such as stromata solitary, cylindrical, sterile apices on top, bright-colored. However, they had different shape and size of perithecia, asci, ascospores, conidiogenous cells, and conidia. The perithecia of *O. bidoupensis* was pyriform to lanceolate and brown-yellow. It was similar to *O. brunneipunctata, O. furcatusubulata,* and *O. langbianensis,* and only *O. houaynhangensis* was clavate.
Multigene phylogeny and morphology of two new species

(Hywel-Jones 1995b; Luangsa-ard et al. 2008; Crous et al. 2018; Lao et al. 2021; Wang et al. 2021a). Conidiogenous cells of *O. bidoupensis* were cone-shaped, forming on hyphae, with a hypertrophic base, tapering abruptly into a thin neck, smooth-walled, with a smaller thin neck (0.42 µm wide) than *O. brunneipunctata* (0.5 µm), *O. furcatosubulata* (0.9 µm), and *O. bouaynhangensis* (0.5 µm).

Due to the unique geographical locations and climate conditions in China and Vietnam, these areas contain a rich species diversity of *Cordyceps* s.l. However, our survey of *Cordyceps* s.l. in China and Vietnam only represented a small portion of the total. More samples of *Cordyceps* s.l. will continue to be collected in China and South-east Asia in order to uncover additional undescribed taxa, and revise species with the incorrect classification position of this group.

**Acknowledgements**

This work was funded by the National Natural Science Foundation of China (31870017, 32060007).

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