

## Article

# Mitogenomics, Phylogeny and Morphology Reveal *Ophiocordyceps pingbianensis* Sp. Nov., an Entomopathogenic Fungus from China

Siqi Chen <sup>1,2,†</sup>, Yuanbing Wang <sup>1,2,†</sup>, Kongfu Zhu <sup>1,2</sup> and Hong Yu <sup>1,2,\*</sup>

<sup>1</sup> Yunnan Herbal Laboratory, College of Ecology and Environmental Science, Yunnan University, Kunming 650091, China; csq2017@mail.ynu.edu.cn (S.C.); wangyb001@126.com (Y.W.); zkf@mail.ynu.edu.cn (K.Z.)

<sup>2</sup> The International Joint Research Center for Sustainable Utilization of Cordyceps Bioresources in China and Southeast Asia, Yunnan University, Kunming 650504, China

\* Correspondence: hongyu@ynu.edu.cn

† These authors contributed equally to this work.

**Abstract:** The new entomopathogenic fungus *Ophiocordyceps pingbianensis*, collected from Southeast China, was described by mitogenomic, morphological, and phylogenetic evidence. The systematic position of *O. pingbianensis* was determined by phylogenetic analyses based on six nuclear gene (ITS, *tef1-α*, *nrSSU*, *nrLSU*, *rpb1* and *rpb2*) and 14 mitochondrial protein-coding gene (PCGs) (*cox1*, *cox2*, *cox3*, *atp6*, *atp8*, *atp9*, *cob*, *nad1*, *nad2*, *nad3*, *nad4*, *nad5*, *nad6* and *nad4L*) data. Phylogenetic analyses reveal that *O. pingbianensis* was belonged to the *Hirsutella nodulosa* clade in the genus *Ophiocordyceps* of Ophiocordycipitaceae. This fungus exhibits distinctive characteristics which differed from other related *Ophiocordyceps* species with slender and geminate stromata, monophialidic conidiogenous cells with an inflated awl-shaped base, a twisty and warty phialide neck and a fusiform or oval conidia, as well as being found on a tiger beetle of Coleoptera buried in moss at the cave. The complete mitochondrial genome of *O. pingbianensis* was a circular DNA molecule 80,359 bp in length, containing 15 PCGs, 24 open reading frames genes (ORFs), 25 transfer RNA genes (tRNAs) and 27 introns. *Ophiocordyceps pingbianensis*, containing 27 introns, has the second largest mitogenome in Ophiocordycipitaceae and was next to *O. sinensis*. To our knowledge, this is the first report of the mitogenome from a new entomopathogenic fungus, and thus provides an important foundation for future studies on taxonomy, genetics and evolutionary biology of Ophiocordycipitaceae.

**Keywords:** mitochondrial genome; new species; phylogenetic analyses; taxonomy



**Citation:** Chen, S.; Wang, Y.; Zhu, K.; Yu, H. Mitogenomics, Phylogeny and Morphology Reveal *Ophiocordyceps pingbianensis* Sp. Nov., an Entomopathogenic Fungus from China. *Life* **2021**, *11*, 686. <https://doi.org/10.3390/life11070686>

Academic Editors: Armin Mešić and Ivana Kušan

Received: 15 June 2021

Accepted: 9 July 2021

Published: 14 July 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

*Ophiocordyceps* Petch was belonged to Ophiocordycipitaceae of Hypocreales [1], erected initially by Petch in 1924. In 1931, Petch defined *O. blattae* Petch as a type species [2]. In this genus, the most famous species *O. sinensis* ((Berk.) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora) is mainly distributed in the high altitudes of the Qinghai-Tibet Plateau [3]. Given the morphological characteristics of asci from several species lacking pronounced apical hemispheric caps and ascospores without being disarticulated into part-spores, *Ophiocordyceps* acts as a subgenus of *Cordyceps* Fr. sensu lato [4–6]. Subsequently, the family Ophiocordycipitaceae was established by the type genus *Ophiocordyceps* [1]. Over the last few years, more than 270 species of *Ophiocordyceps* have been described, making it the largest genus in Ophiocordycipitaceae [1,7–13]. *Ophiocordyceps* is characterized by a darkly pigmented and tough, wiry, fibrous or pliant stromata ordinarily or obliquely arranged superficial to immersed perithecia; ascospores are commonly cylindrical and multi-septate, disarticulated into part-spores or non-disarticulating [1,14,15].

Asexual morphs of *Ophiocordyceps* consist of *Hirsutella* Pat., *Hymenostilbe* Petch, *Paraisaria* Samson and B.L. Brady, *Sorosporella* Sorokin, *Stilbella* Lindau, as well as *Syngliocladium*

Petch [1,14]. The main anamorph of *Ophiocordyceps* refers to *Hirsutella*. The fungal genus *Hirsutella* was a pathogen of insects, mites and nematodes, which was erected by Patouillard in 1892, and *Hirsutella* was initially described by Petch in 1924. In 1998, Hodge introduced the monograph and molecular phylogeny of *Hirsutella*. To be specific, she reviewed the taxonomy of *Hirsutella* species and described 68 species to pertain to *Hirsutella* [16]. *Hirsutella* was considered to show relationships to the genus *Ophiocordyceps* typified by a sexual morph [1]. The morphological characteristics of this genus was considered to produce basally inflated phialides, tapering towards the apex, discontinuous hymenial layer and conidia embedded in a mucous sheath [15,17,18].

The mitochondrion acts as a master regulator of metabolism. As DNA sequencing technology leaps forward, the mitochondrial DNA (mtDNA) is recognized as an effective marker for phylogenetic analysis, with the advantage of exhibiting a faster mutation rate than nuclear DNA [19]. Moreover, mtDNA is also appropriate for phylogenetic analysis due to its high copy number and conservative gene functions [20].

Furthermore, there are differences in the size of fungal mitochondria in different species. From the current reports, the smallest mitogenome known in the fungi was 12.1 kb in *Rozella allomycetis* (Doweld) Letcher in Cryptomycota [21], while the largest was 272,238 bp in *Morchella importuna* M. Kuo, O'Donnell and T.J. Volk in Pezizomycetes [22]. The mitochondrial genome of the Ophiocordycipitaceae species is like an obvious closed circular structure, comprising naked double stranded DNA. It commonly contains 15 protein-coding genes (e.g., 3 subunits of cytochrome c oxidase (cox1–3), 3 subunits of ATP synthase (*atp6*, 8 and 9), cytochrome b gene (*cob*), 7 subunits of NADH dehydrogenase (*nad1–6*, *nad4L*) and 1 ribosomal protein S3 (*rps3*), as well as ribosomal RNA genes (*rnl*, *rns*) and transfer RNA genes (*trn*). Thus far, mitogenomes of only ten species in Ophiocordycipitaceae have been described, in which mitogenomes of *Hirsutella* species have been rarely reported. The existing sequence data of mitogenome from NCBI only include *H. minnesotensis* Sen Y. Chen, Xing Z. Liu and F.J. Chen, *H. rhossiliensis* Minter and B.L. Brady, *H. thompsonii* F.E. Fisher and *H. vermicola* M.C. Xiang and Xing Z. Liu. The largest mitogenome known reached 157.5 kb in *O. sinensis* [23], and the smallest was 52,245 bp in *H. minnesotensis* [24]. Different variations of mitogenome sizes of hypocrealean fungi were largely attributed to differences in the number/length of introns and the length of intergenic regions, and the mentioned introns most likely were obtained through horizontal transfer from other fungal species [25].

In the present study, an unknown species of *Ophiocordyceps* attacking a larva of tiger beetle was collected from Daweishan National Nature Reserve, Pingbian County, Yunnan Province, China. Mitogenomics, phylogeny and morphology of this fungus were determined, and its systematic position was established in Ophiocordycipitaceae. It was revealed a new species of *Ophiocordyceps* with a *Hirsutella* morph. The complete mitogenome of the new fungus was sequenced, assembled and annotated. Genome structures, gene contents, codon usage and gene arrangement were analyzed.

## 2. Materials and Methods

### 2.1. Fungal Materials and Isolation

In this study, live specimens were collected on a tiger beetle of Coleoptera buried in the moss in a cave in Daweishan National Nature Reserve, Pingbian County, Yunnan Province, China. Before examination, the specimen was transferred to a laboratory and then stored at 4 °C. To detect and further study the culture, stromata were photographed and then measured with an Olympus SZ61 stereomicroscope. A stroma of the fungus growing from the beetle larva fell to small segments nearly 5 mm long. The respective segment was surface-sterilized in 30% H<sub>2</sub>O<sub>2</sub> for 5 min, then soaked in 70% alcohol for 2 min and subsequently rinsed sufficiently in sterilized water, and lastly dried on the sterilized filter paper. Afterwards, the segments were placed on Potato Dextrose Agar (PDA) plates. The specimen was deposited in Yunnan Herbal Herbarium (YHH), Yunnan University.

The cultures of this fungus were deposited in Yunnan Fungal Culture Collection (YFCC), Yunnan University.

## 2.2. Morphological Observations

The fresh specimen, including stromata and host, were photographed with Olympus CX40 and BX53 microscopes. The colonies on PDA plates were cultured at 4 °C for 4 weeks, and the colonies characteristics (size, texture and color) were photographed with the Cannon 700D camera to characterize the morphology of colonies. For to asexual morphological descriptions, microscope slide cultures were prepared by placing a small amount of mycelia on 5-mm diameter PDA medium blocks that were overlaid by a cover slip. The cultures on the slants were transferred to PDA plates and then cultured with an incubator for 25 d at 4 °C. Next, the colonies were photographed and then measured every four days. The micro-morphological observations and measurements were performed under Olympus CX40 and BX53 microscopes, as well as a FEI QUANTA200 scanning electron microscope [26].

## 2.3. DNA Extraction, PCR and Sequencing of Nuclear Genes

Axenic living cultures of the new species were collected from a PDA plate to prepare for DNA extraction. The DNA extraction was performed with the CTAB method in Liu et al. [27]. The nuclear ribosomal small subunit (*nrSSU*) was amplified with the primer pair *nrSSU*-CoF and *nrSSU*-CoR [28]. The nuclear ribosomal large subunit (*nrLSU*) and translation elongation factor 1 $\alpha$  (*tef-1 $\alpha$* ) were amplified with the primers LR5 and LR0R [29,30] and EF1 $\alpha$ -EF and EF1 $\alpha$ -ER [1,30], respectively. To amplify the largest subunits of RNA polymerase II (*rpb1*) (*rpb2*), the primer pair RPB1-5'F and RPB1-5'R, as well as the primer pair RPB2-5'F and RPB2-5'R, were applied respectively [31]. The nuclear ribosomal internal transcribed spacer region (ITS) was amplified using the primer pair ITS4 and ITS5 [32]. The polymerase chain reaction (PCR) assay was performed as described by Wang et al. [33]. PCR products were sequenced with an automatic sequence analyzer (BGI). When PCR products could not be sequenced directly, cloning was performed with the TaKaRa PMD<sup>TM</sup>18-T vector system (TaKaRa Biotechnology, Dalian, China).

## 2.4. Sequencing, Assembly and Annotation of Mitogenome

After the DNA extraction and the quality detection, the extracted genomic DNA was transported on dry ice for high-throughput sequencing at Novogene Co., Ltd. (Beijing, China) by applying dry ice for high-throughput sequencing. Following standard procedures, the extracted genomic DNA was sequenced on the Illumina HiSeq 3000 platform using a 350 bp paired-end reads, which resulted in 3 GB of data and 100 $\times$  sequencing depth. Using the high-throughput sequencing data, mitogenome of the new species was assembled with the software SPAdes v. 3.11.0 [34], which involved three processes, i.e., (1) initial splicing, (2) contig screening and extension and (3) hole filling and re-splicing. The complete mitogenome of the new species was annotated by combining the results of a MFannot tool [35] and an ARWEN web server, with artificial correction. The content of the annotated sample mainly included the protein coding genes (PCGs), the rRNA genes, the tRNA genes and the open reading frames (ORFs). Next, the graphical map of the complete mitogenome of the new species was generated with the software Organellar Genome DRAW tool [36].

## 2.5. Sequence Analyses of Mitogenome

The GC content of the mitochondrion sequence was analyzed using Mega 7. tRNA genes were analyzed with tRNAscan-SE 2.0 (<http://lowelab.ucsc.edu/tRNAscan-SE/index.html>, accessed on 1 October 2020). Sequence Manipulation Suite ([http://www.detaibio.com/sms2/codon\\_usage.html](http://www.detaibio.com/sms2/codon_usage.html), accessed on 20 October 2020) and CodonW 1.4.4 were adopted to calculate codon usage in the mitogenome. Genomic synteny analysis of six *Ophiocoryceps* species was conducted using Mauve v2.4.0 (A.C. Darling, 2004).

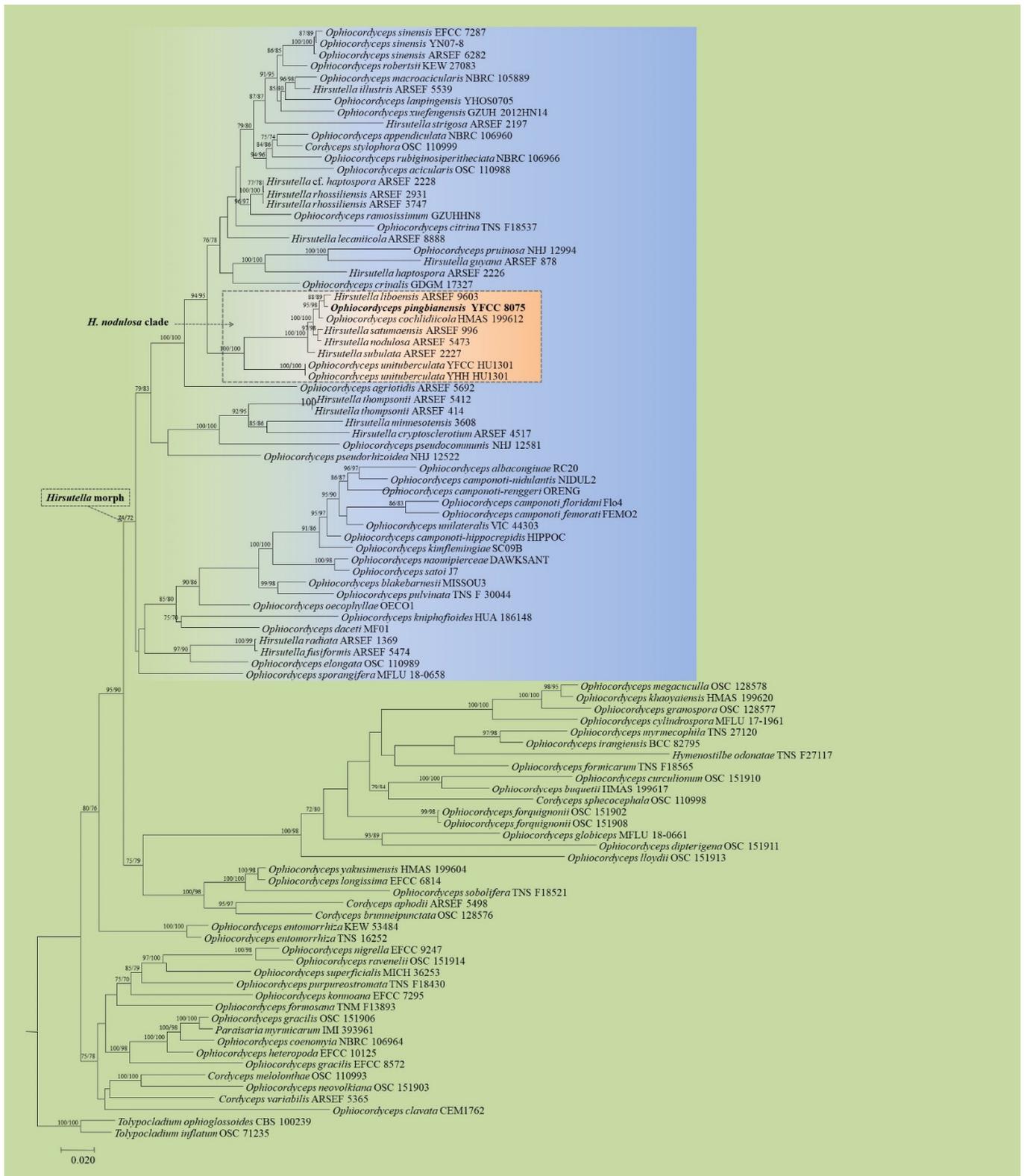
## 2.6. Phylogenetic Analyses

Raw trace files were inspected and edited using MEGA7. Two species of *Tolypocladium inflatum* W. Gams and *T. ophioglossoides* J.F. Gmel were designated as the outgroup to root the trees. Five gene (*nrSSU*, *nrLSU*, *tef-1 $\alpha$* , *rpb1* and *rpb2*) sequences of *Hirsutella*, *Ophiocordyceps* and the outgroup, were retrieved from GenBank. The majority of ITS sequences of *Hirsutella* were also retrieved from GenBank. Some manual adjustments were made as the *nrSSU* gene contained an intron (Table S1). The sequences were aligned with the programmes Clustal X2.0 and MEGA7 [37,38]. Phylogenetic analyses were performed by combining the concatenated 14 PCGs sequences generated here with other mitogenomic sequences of species in Hypocreales downloaded from NCBI. *Neurospora crassa* Shear and B.O. Dodge and *Podospira anserine* Rabenh were designated as the outgroup. The 14 mitochondrial PCGs were aligned using MUSCLE. [39]. Maximum likelihood (ML) and Bayesian Inference (BI) phylogenetic trees were estimated using RaxML 7.0.3 and MrBayes v.3.1.2. [26,40,41].

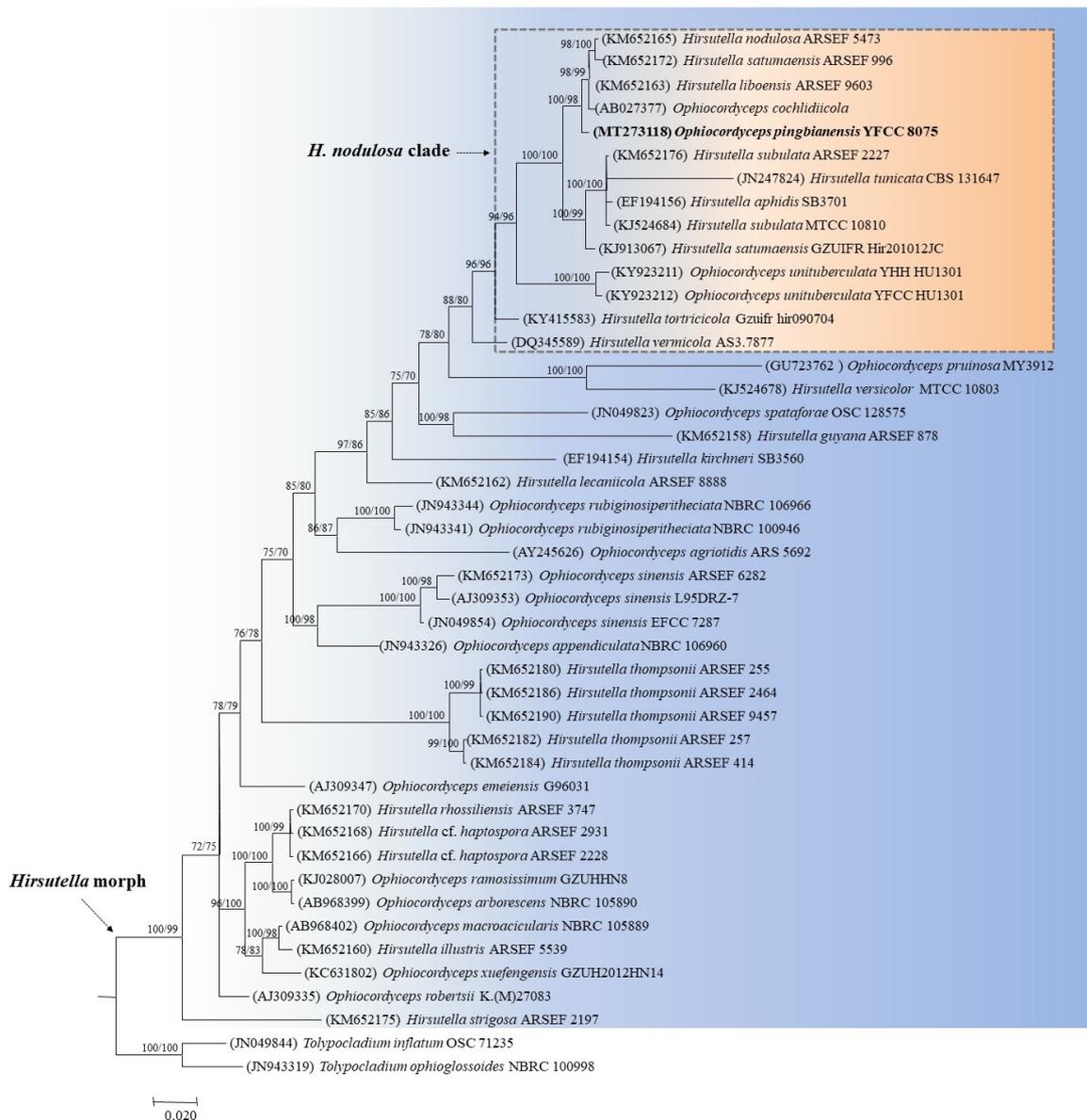
## 3. Results

### 3.1. Phylogenetic Analyses Based on Nuclear Genes

For the ML and BI trees estimated for the five nuclear genes, the new species was closely related to *H. liboensis* X. Zou, A.Y. Liu and Z.Q. Liang and *O. cochliidiicola* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora, clustered as a separate clade from other allied species in the genus *Ophiocordyceps* (Figure 1). For the ML and BI trees estimated for the ITS sequences, there was no significant difference in topology between the five-gene and ITS phylogenetic trees. The new species formed an independent clade from its allied species of *Hirsutella* and was closely grouped with *H. liboensis* and *O. cochliidiicola* (Figure 2).



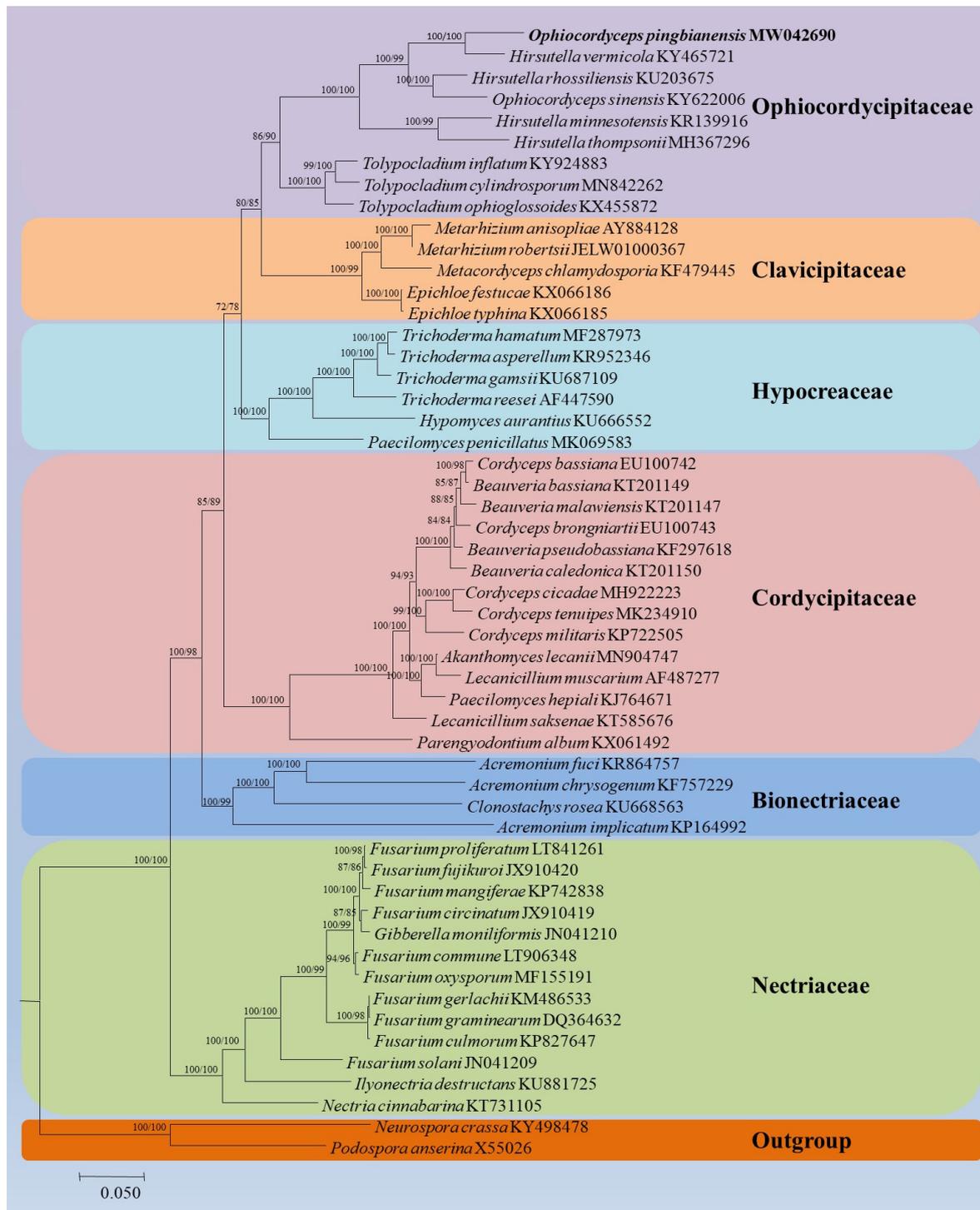
**Figure 1.** Phylogenetic tree of *Ophiocordyceps* inferred from Maximum Likelihood (ML) and Bayesian Inference (BI) analyses based on a five-gene (*nrSSU*, *nrLSU*, *tef-1 $\alpha$* , *rpb1* and *rpb2*) dataset. Values at the nodes before and after the backslash are ML bootstrap proportions and BI posterior probabilities, respectively. Support values greater than 50% are indicated at the nodes. Phylogenetic tree shows the placement of *O. pingbianensis* within the genus *Ophiocordyceps*. *Tolypocladium inflatum* and *T. ophioglossoides* are designed as the outgroup.



**Figure 2.** Phylogenetic relationships of *Ophiocordyceps pingbianensis* to the related taxa from ML and BI analyses based on ITS sequences. Values at the nodes before and after the backslash are ML bootstrap proportions and BI posterior probabilities, respectively. Support values greater than 50% are indicated at the nodes. Phylogenetic tree shows that *O. pingbianensis* belongs to the *H. nodulosa* clade in the genus *Ophiocordyceps* with *Hirsutella* morph. *Tohyopcladium inflatum* and *T. ophioglossoides* were designed as the outgroup. GenBank accession numbers for ITS sequences are placed in the bracket before the taxon name.

### 3.2. Phylogenetic Analysis Based on Mitochondrial Genes

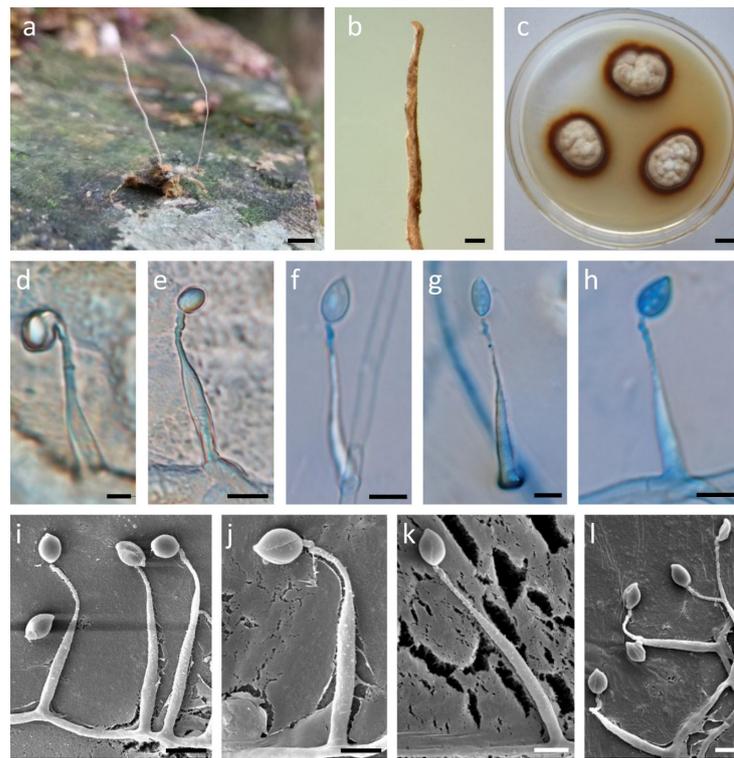
The ML tree and BI tree were estimated for the mitochondrial PCG dataset of 52 species in Ascomycota from GenBank (Table S2). The six well-supported clades of Hypocreales were recognized by ML and BI analyses, including species of the families Bionectriaceae, Clavicipitaceae, Cordycipitaceae, Hypocreaceae, Nectriaceae and Ophiocordycipitaceae (Figure 3). As indicated from the phylogenetic analysis, the new species was a member of the family Ophiocordycipitaceae. The new species was clustered together with *H. minnesotensis* and *H. vermicola* in the genus *Ophiocordyceps* of Ophiocordycipitaceae.



**Figure 3.** Phylogenetic relationships among 53 taxa of Sordariomycetes based on ML and BI analyses from 14 concatenated mitochondrial protein-coding genes (PCGs). The 14 PCGs included subunits of the respiratory chain complexes (*cob*, *cox1*, *cox2*, *cox3*), ATPase subunits (*atp6*, *atp8*, *atp9*), NADH: quinone reductase subunits (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*). Values at the nodes before and after the backslash are ML bootstrap proportions and BI posterior probabilities, respectively. Support values greater than 50% are indicated at the nodes. Phylogenetic tree shows the placement of *O. pingbianensis* in the family Ophiocordycipitaceae. *Neurospora crassa* and *Podospora anserina* were designed as the outgroup.

### 3.3. Taxonomy

*Ophiocordyceps pingbianensis* H. Yu, S.Q. Chen and Y.B. Wang, sp. nov. (Figure 4)



**Figure 4.** Ecology and morphology of *Ophiocordyceps pingbianensis*. (a) Stromata arising from a Larva of tiger beetle of Coleoptera; (b) The tip of a stroma; (c) Colony on PDA medium; (d–l) Conidiogenous cells. Scale bars: (a) = 0.5 cm; (b) = 500  $\mu\text{m}$ ; (c) = 1 cm; (d–l) = 5  $\mu\text{m}$ .

**MycoBank:** MB 840055.

**Etymology:** Named after the location Pingbian County where this species was collected.

**Holotype:** China. Yunnan Province: Pingbian County, Daweishan National Nature Reserve, at 103°41'57" E, 22°57'45" N, alt. 1536 m, on a tiger beetle of Coleoptera buried in the moss in a cave, 17 June 2019, Y.B. Wang (YHH 18766, holotype; YFCC 8075, ex-holotype living culture).

Stromata geminate, slender, smooth and unbranched, flexible to ligneous, with surface color from light deep yellow to dark brown, 17–21 ( $\bar{x}$  = 19) mm in length, 0.16–0.21 ( $\bar{x}$  = 0.19) mm in width. Colonies on PDA slow-growing, with a diam up to 18–20 mm after 4 weeks at 4 °C, lanate, consisting of a loose mycelial felt with annulation, beige to pale brown, dark brown at the edge. After 90 d of culture, stromata were not observed. Hyphae hyaline, septate, branched, smooth-walled, 2.8–4.4  $\mu\text{m}$  ( $\bar{x}$  = 3.6) in width. Conidiogenous cells monophialidic, non-septate, arising from hyphae laterally, with an inflated awl-shaped base, 20.4–31.6  $\mu\text{m}$  ( $\bar{x}$  = 26.0) long, 3.2–5.2  $\mu\text{m}$  ( $\bar{x}$  = 4.2) wide at the base, phialide neck twisty and warty, 0.6–1.4  $\mu\text{m}$  ( $\bar{x}$  = 1.0) wide at the apex. Conidia solitary, hyaline, aseptate, smooth-walled, fusiform or oval, 3.1–5.3  $\times$  4.6–7.5  $\mu\text{m}$  ( $\bar{x}$  = 3.9  $\times$  6.4), embedded in a mucous sheath of 6.5–8.6  $\times$  7.1–9.2  $\mu\text{m}$  ( $\bar{x}$  = 6.8  $\times$  8.9).

**Host:** Larva of a tiger beetle (Coleoptera).

**Habitat:** Buried in the moss in a cave.

**Type locality:** Daweishan National Nature Reserve, Pingbian County, Yunnan Province, China.

**Sexual stage:** The fruiting body was not observed.

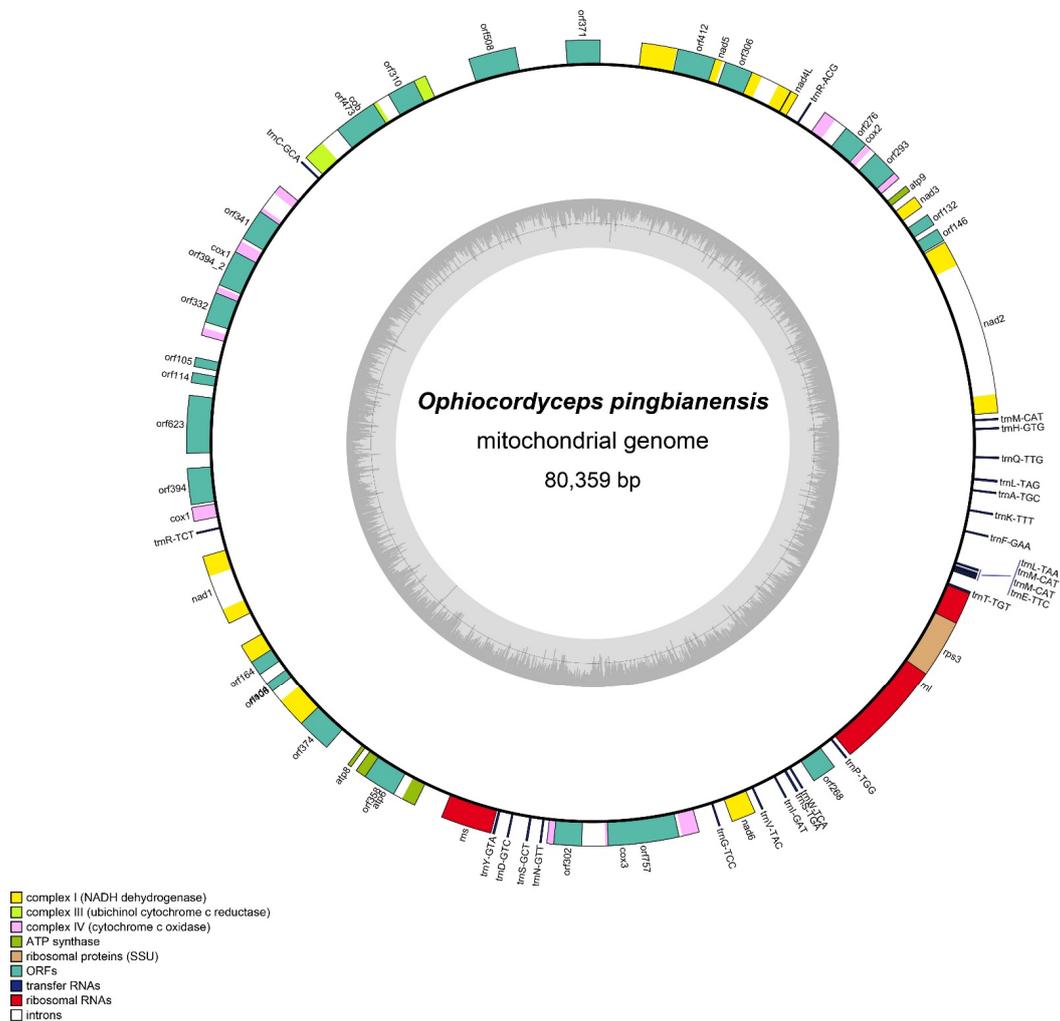
**Notes:** *Ophiocordyceps pingbianensis* is characterized by geminate stromata, slender, unbranched and a surface color from light deep yellow to dark brown; monophialidic conidiogenous cells with an inflated awl-shaped base, twisty and warty phialide neck, fusiform or oval conidia. Morphological comparison showed that *O. pingbianensis* was distinct from other closely related species (Table 1), which was concordant with molecular phylogenetic results.

**Table 1.** A morphological comparison of *Ophiocordyceps pingbianensis* and its related species.

Species	Host	Habitat	Synnemata/Stromata	Conidiogenous Cells	Conidia	References
<i>H. aphidis</i>	Aphids	On plant	Small or as short cushions	Narrowly conical, length (17–90 $\mu\text{m}$ ), neck width (0.6–1.0 $\mu\text{m}$ ), base width (2.6–4.0 $\mu\text{m}$ )	Slightly crescent-shaped or cymbiform, usually in pairs, 7.0–18.3 $\times$ 2.8–4.6 $\mu\text{m}$ , with mucous sheath	[42]
<i>H. liboensis</i>	Larvae of Cossidae (Lepidoptera)	In tree hole	Clustered	Base significantly swollen, length (28–30 $\mu\text{m}$ ), neck width (1–2 $\mu\text{m}$ ), twisting in 2–3 helices at the apex, base width (3–4.5 $\mu\text{m}$ )	Fusiform or orange-segmented, in pairs or single, 6–8 $\times$ 3–5 $\mu\text{m}$ , with mucous sheath	[43]
<i>H. nodulosa</i>	Lepidoptera, Acari	On plant	None	Base swollen, length (20–35 $\mu\text{m}$ ), base width (4 $\mu\text{m}$ ), neck often twists in a helix at the apex, neck width (1 $\mu\text{m}$ ), with tiny warts	Ellipsoid or like orange segments, 5–6 $\times$ 3 $\mu\text{m}$ , with mucous sheath	[44]
<i>O. pingbianensis</i>	Larvae of tiger beetle (Coleoptera)	Clinging to fallen leaves	Geminate, 17–21 $\times$ 0.16–0.21	Base obviously swollen, length (20.4–31.6 $\mu\text{m}$ ), base width (3.2–5.2 $\mu\text{m}$ ), neck width (1–1.5 $\mu\text{m}$ ), twisty and warty at the apex	Solitary, fusiform or oval, 5.3–7.5 $\mu\text{m}$ long, 3.14.6 $\mu\text{m}$ wide, with mucous sheath	This study
<i>H. satumaensis</i>	Larvae of <i>Bombyx mori</i> (Lepidoptera)	On plant	Clustered, 3.0–6.5 $\times$ 0.5 mm	With conoid to cylindrical base, size (5–17 $\times$ 3–4.5 $\mu\text{m}$ ), neck length (7 $\mu\text{m}$ ), twisting in a helix at the apex; base with warts	Fusiform or like orange segments, 5–7.5 $\times$ 3–5 $\mu\text{m}$ , with mucous sheath	[14,45]
<i>H. subuluta</i>	Larvae of Lepidoptera	On plant	Solitary, 15–50 $\times$ 0.1–0.3 mm	Phialidic, neck 6–12 $\mu\text{m}$ long, base size (4–8 $\times$ 3–5 $\mu\text{m}$ )	Narrowly ellipsoid, in pairs or single, 4–8 $\times$ 1.5–2.5 $\mu\text{m}$ , with mucous sheath.	[16]
<i>H. tortricicola</i>	Larvae of Tortricidae (Lepidoptera)	In wilted leaf	Solitary	Base obviously swollen, length (18–22 $\mu\text{m}$ ), base width (3.5–4 $\mu\text{m}$ ), neck width (1–1.5 $\mu\text{m}$ ), neck often twists in 1–2 helices at the apex	Ellipsoid or like orange segments, in pairs or single, 2.7–3.6 $\times$ 1.4–1.8 $\mu\text{m}$ , with mucous sheath	[46]
<i>O. unituberculata</i>	Larvae of Noctuidae (Lepidoptera)	In soil or cling to the fallen leaves	Clustered, 5–76 $\times$ 0.4–0.7 mm	With an inflated awl-shaped base, length (31.9–128.3 $\mu\text{m}$ ), base width (1.8–5.0 $\mu\text{m}$ ), neck width (0.5–1.2 $\mu\text{m}$ ). With a large periclinal protuberance near the apex	Solitary, lanceolate to fusiform, 6.3–10.6 $\times$ 1.9–3.7 $\mu\text{m}$ , with mucous sheath	[18]
<i>H. vermicola</i>	Bacteria-feeding nematodes	In soil	None	Base obviously swollen, length (14–26 $\mu\text{m}$ ), base width (3.0–5.0 $\mu\text{m}$ ), neck width (1–2 $\mu\text{m}$ ), neck often twists in a helix towards the apex	More or less ellipsoid, single or in groups of 2–8 $\times$ 3–5 $\mu\text{m}$ , with mucous sheath	[47]

### 3.4. Mitogenomic Characteristics of *Ophiocordyceps Pingbianensis*

The complete mitogenome of *O. pingbianensis* was an obvious circular molecule with a length of 80,359 bp (Figure 5). It had an overall GC content of 29.89%. The mitogenome of *O. pingbianensis* was a rather high capacity and compacted genome with genic regions (including intronic regions) accounting for 71.54% and intergenic regions taking up 27%. The protein-coding regions took up 92.19% of the whole mitogenome of *O. pingbianensis*, containing 2 rRNA genes (i.e., *rnl* and *rns*) genes, 15 standard protein-coding genes (PCGs), 24 ORFs, 25 transfer genes (tRNAs) (Table S3). The sizes of *rns* and *rnl* were 1652 bp and 6444 bp, respectively. The total length of the 15 PCGs was 14682 bp, including 3 subunits of cytochrome c oxidase (*cox1*, *cox2* and *cox3*), 3 subunits of ATP synthase (*atp6*, *atp8* and *atp9*), one cytochrome b gene (*cob*) and seven subunits of NADH dehydrogenase complex (*nad1*, *nad2*, *nad3*, *nad4*, *nad5*, *nad6* and *nad4L*) and 1 ribosomal protein S3 (*rps3*). The lengths of 25 tRNAs ranged from 69 to 84 bp and 24 ORFs (*orf146*, *orf132*, *orf293*, *orf276*, *orf306*, *orf412*, *orf371*, *orf508*, *orf310*, *orf473*, *orf341*, *orf394\_2*, *orf332*, *orf105*, *orf114*, *orf623*, *orf394*, *orf164*, *orf108*, *orf374*, *orf358*, *orf302*, *orf757* and *orf268*).



**Figure 5.** The mitogenomic circular map of the complete mitochondrial genome of *Ophiocordyceps pingbianensis*. Genes are represented with different color blocks.

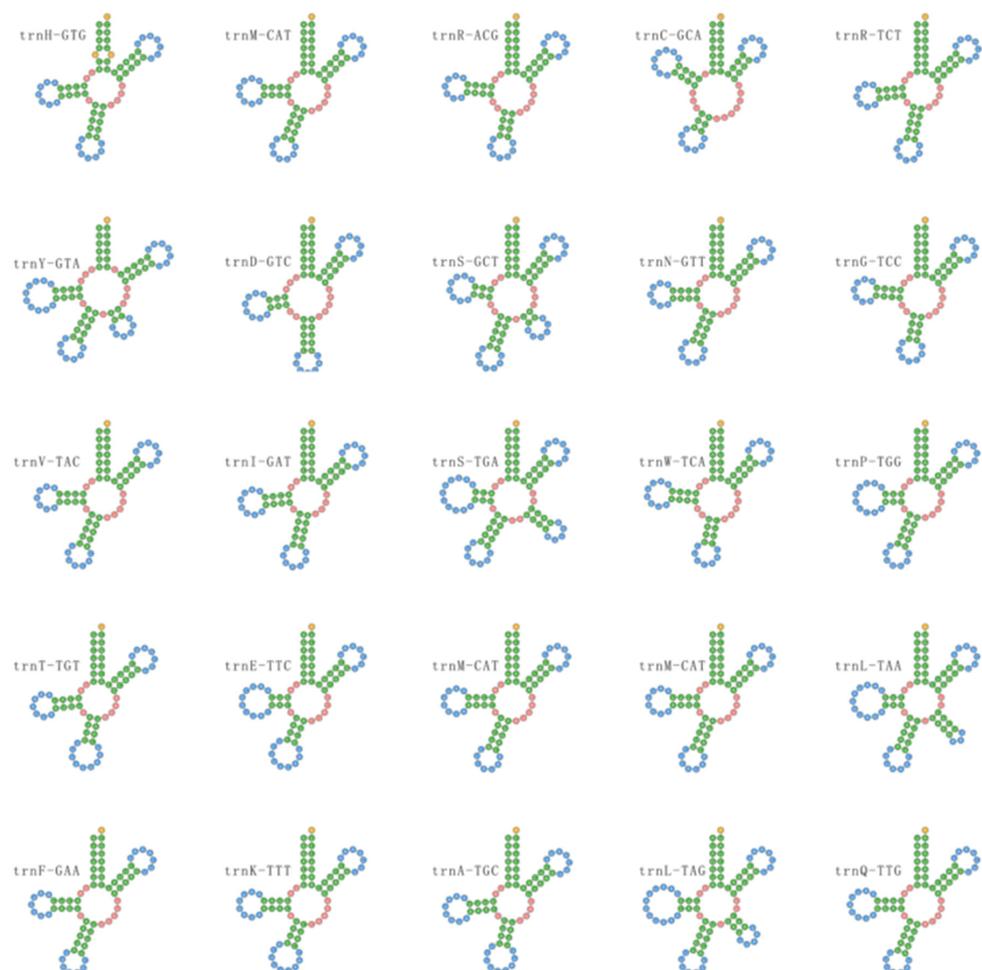
### 3.5. Codon Usage

The codon usage of mitochondrial genes (*atp6–9*, *cob*, *cox1–3*, *nad1–6*, *nad4L*, *orf146*, *orf132*, *orf293*, *orf276*, *orf306*, *orf412*, *orf371*, *orf508*, *orf310*, *orf473*, *orf341*, *orf394\_2*, *orf332*, *orf105*, *orf114*, *orf623*, *orf394*, *orf164*, *orf108*, *orf374*, *orf358*, *orf302*, *orf757* and *orf268*) of

*O. pingbianensis* was determined. The frequency of initiation codon ATG was the highest, followed by AAA, *orf473*, *orf394\_2*, *orf332* and *orf302* started with “AAA”, *orf146*, *orf293* and *orf341* started with “AGA”, *orf164* and *orf358* started with “AAC”. The frequency of stop codon TAA was the highest, followed by TAG. Only *cox1* was terminated by “GCT” (Table S4). Leucine was the most common amino acid in the PCGs in the mitogenome of *O. pingbianensis*, followed by Arginine (Table S5). For codon usage, the most commonly used codons were UUA (3.88%), AGA (3.6%), CCU (2.37%), GCU (1.99%), UCU (1.94%), and GGU (1.93%), and the least used codons included CUG (0.19%), AUC (0.18%), ACC (0.17%), CGG (0.15%) and CUC (0.11%).

### 3.6. Transfer RNAs

In the mitogenome of *O. pingbianensis*, the 25 tRNAs coded for all 20 amino acids (Table S6). As indicated from the results, all tRNAs adopt a typical cloverleaf structure except for *trnS-GCT*, *trnY-GTA*, *trnL-TAA*, *trnL-TAG* and *trnS-TGA* (Figure 6). As shown in Table S6, some of the 25 tRNAs existed in multiple copies, the *trnM-CAT* gene for methionine appeared in three copies. Two different tRNAs for arginine (*trnR-ACG* and *trnR-TCT*), Serine (*trnS-GCT* and *trnS-TGA*) and Leucine (*trnL-TAA* and *trnL-TAG*) were found.



**Figure 6.** The predicted tRNA structures of *Ophiocordyceps pingbianensis*. Secondary structures predicted by tRNA scan-SE show that all tRNAs adopt a typical cloverleaf structure except for *trnS-GCT*, *trnY-GTA*, *trnL-TAA*, *trnL-TAG* and *trnS-TGA*.

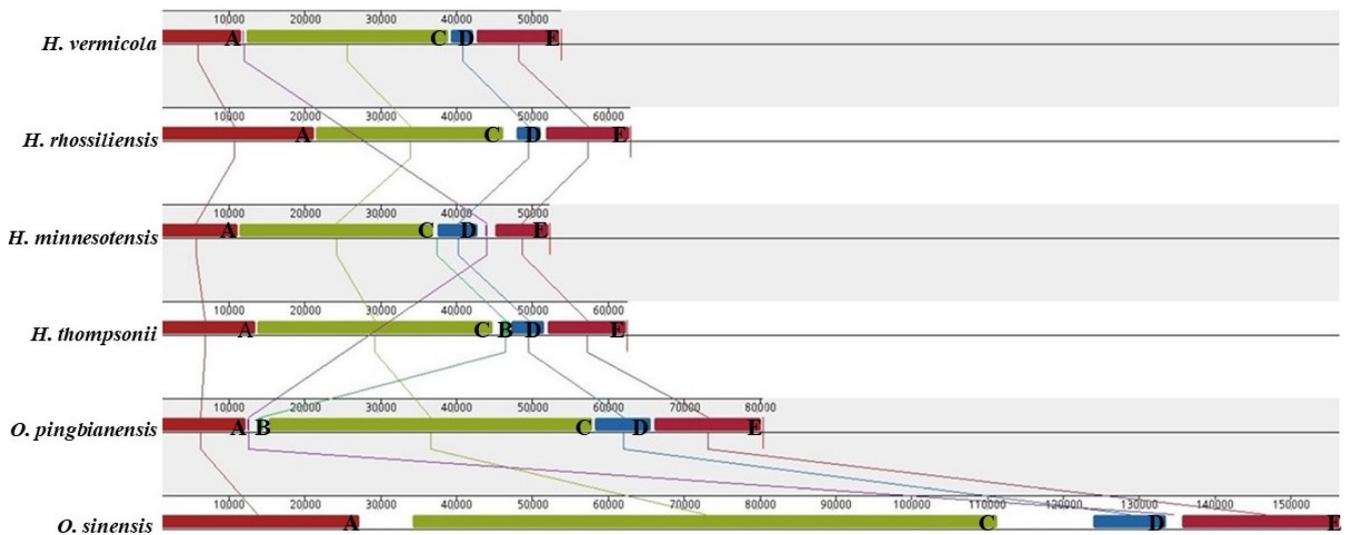
### 3.7. Gene Arrangement Analysis

Overall, the relative positions of most PCGs and tRNAs were relatively conservative across the six *Ophiocordyceps* species (Figure 7). However, several tRNAs and PCGs were

distributed in different positions in six mitogenomes, showing the variability of the relative gene order of these mitogenomes. As revealed from the results of genomic synteny (Figure 8), the six *Ophiocordyceps* mitogenomes fell to 4–5 homologous regions. Homologous regions were identified in the mitogenome of *O. pingbianensis* and *H. minnesotensis*, adding the homologous region B. However, there was an absence of the homologous region B in mitogenomes of *O. sinensis*, *H. thompsonii*, *H. vermicola* and *H. rhossiliensis*. The relevant orders of four homologous regions A, C, D and E were conserved across the six *Ophiocordyceps* mitogenomes. The homologous regions B was distributed in different positions of all the mitogenomes.

<i>O. pingbianensis</i>	<i>H. minnesotensis</i>	<i>H. rhossiliensis</i>	<i>H. thompsonii</i>	<i>H. vermicola</i>	<i>O. sinensis</i>
rnl	ml	rnl	rnl	rnl	ml
trnT-TGT	rps3	trnT-TGT	trnT-TGT	trnT-TGT	rps3
trnE-TTC	trnT-TGT	trnE-TTC	trnE-TTC	trnE-TTC	trnT-TGT
trnM-CAT	trnE-TTC	trnM-CAT	trnM-CAT	trnM-CAT	trnG-TTC
trnM-CAT	trnM-CAT	trnM-CAT	trnM-CAT	trnM-CAT	trnM-CAT
trnL-TAA	trnM-CAT	trnF-GAA	trnL-GAA	trnL-GAA	trnM-CAT
trnF-GAA	trnL-TAA	trnL-TAA	trnA-TGC	trnF-GAA	trnL-TAA
trnK-TTT	trnA-TGC	trnF-GAA	trnK-TTT	trnK-TTT	trnP-GAA
trnA-TGC	trnF-GAA	trnK-TTT	trnK-TTT	trnA-TGC	trnL-TTT
trnL-TAG	trnK-TTT	trnA-TGC	trnL-TAG	trnL-TAG	trnA-TGC
trnQ-TTG	trnL-TAG	trnL-TAG	trnQ-TTG	trnQ-TTG	trnG-TCC
trnH-GTG	trnQ-TTG	trnQ-TTG	trnH-GTG	trnH-GTG	trnL-TAG
trnM-CAT	trnH-GTG	trnH-GTG	trnM-CAT	trnM-CAT	trnG-TTG
nad2	trnM-CAT	trnM-CAT	nad2	nad2	trnH-GTG
nad3	nad2	nad2	nad3	nad3	trnM-CAT
atp9	nad3	nad3	atp9	atp9	nad2
cox2	atp9	atp9	cox2	cox2	nad3
trnR-ACG	cox2	cox2	trnR-ACG	trnR-ACG	atp9
nad4L	trnR-ACG	trnR-ACG	nad4L	nad4L	cox2
nad5	nad4L	nad4L	nad5	nad5	trnA-ACG
cob	nad5	nad5	cob	cob	nad4L
trnC-GCA	cob	cob	trnC-GCA	trnC-GCA	nad5
cox1	trnC-GCA	trnC-GCA	cox1	cox1	cob
trnR-TCT	cox1	cox1	trnR-TCT	trnR-TCT	trnC-GCA
nad1	trnR-TCT	trnR-TCT	trnC-GCA	nad1	cox1
nad4	nad1	nad1	nad1	nad4	trnA-TCT
atp8	nad4	nad4	nad4	atp8	nad1
atp6	atp8	atp8	atp8	atp6	nad4
rns	atp6	atp6	atp6	rns	atp8
trnY-GTA	rns	rns	rns	trnY-GTA	atp6
trnD-GTC	trnY-GTA	trnY-GTA	trnY-GTA	trnD-GTC	rns
trnS-GCT	trnD-GTC	trnD-GTC	trnD-GTC	trnS-GCT	trnT-GTA
trnN-GTT	trnS-GCT	trnS-GCT	trnS-GCT	trnN-GTT	trnA-GTC
cox3	trnN-GTT	trnN-GTT	trnN-GTT	cox3	trnS-GCT
trnG-TCC	cox3	cox3	cox3	trnG-TCC	trnA-GTT
nad6	trnG-TCC	trnG-TCC	trnG-ACC	nad6	cox3
trnV-TAC	nad6	nad6	trnG-TCC	trnV-TAC	trnI-TAT
trnI-GAT	trnV-TAC	trnV-TAC	nad6	trnI-GAT	trnG-TCC
trnS-TGA	trnI-GAT	trnI-GAT	trnV-TAC	trnS-TGA	nad6
trnW-TCA	trnS-TGA	trnS-TGA	trnI-GAT	trnW-TCA	trnV-TAC
trnP-TGG	trnW-TCA	trnW-TAC	trnS-TGA	trnP-TGG	trnI-GAT
rps3	trnP-TGG	trnP-TGG	trnW-TCA		trnS-TGA
			trnP-TGG		trnT-TCA
					trnP-TGG

Figure 7. A comparison of gene order among six *Ophiocordyceps* mitogenomes. The genes are colored on the basis of their functional groups. The non-coding region (NCR) is not indicated.



**Figure 8.** Mitogenome collinearity analysis of six *Ophiocordyceps* species. The progressive Mauve alignment shows the homologous regions shared among the mitogenomes, and it also connected these regions with lines, indicating their corresponding positions among the homologous regions in order to visualize the gene arrangement. (A–E) represent different homologous regions.

#### 4. Discussion

By morphological and molecular phylogenetic researches, *O. pingbianensis* was found as a novel species, being belonged to the *H. nodulosa* clade. Morphological differences still exist between *O. pingbianensis* and its related species, such as the conidiogenous cells of *H. liboensis* with being smooth and polyphialidic [43]. *O. pingbianensis* has a warty phialide neck of conidiogenous cells, whereas a larger periclinal protuberance was produced near the apex of conidiogenous cells of *O. unituberculata* H. Yu, Y.B. Wang and Y.D. Dai [18]. Conidia of *O. pingbianensis* were smooth, fusiform or oval, inconsistent with *O. unituberculata* with larger lanceolate to fusiform conidia and *H. nodulosa* with non-mucoid conidia. The host of *O. pingbianensis* was a tiger beetle of Coleoptera; however, these hosts of its closely related species *H. satumaensis*, *H. nodulosa*, *H. liboensis* and *O. cochliidiicola* are Lepidoptera.

The mitogenome of *O. pingbianensis* was a circular DNA molecule with a length of 80,359 bp, and the mitogenome size was the second largest within the mitogenomes of the Ophiocordycipitaceae species reported. The mitogenome sizes were different among species in *Ophiocordyceps* attributed to differences in the number of introns. For instance, the *H. thompsonii* mitogenome exhibited 15 introns [25], the *H. rhossiliensis* mitogenome had 13 introns [48], the *H. minnesotensis* mitogenome had 13 introns [24] and the *H. vermicola* mitogenome exhibited 7 introns [47]. The mitogenome size of *O. pingbianensis* was the second largest because it contained 27 introns. The mitochondrial genome of *H. minnesotensis* exhibited significant intron degeneration for the *nad4L* and *cox1-i1* genes, which might be caused by unexpected stop codons or frame shifting [24]. In *H. rhossiliensis*, there were 10 group I introns and one unclassified intron in six genes (i.e., *rnl*, *cob*, *cox1*, *cox3*, *nad1* and *nad5*) [48]. This indicated an intron presence/absence dynamics cause mitogenome size variations in the *Ophiocordyceps* species. On the whole, 27 ORFs were identified in *O. pingbianensis*, and except for *orf144*, *orf131*, all ORFs were transcribed at the identical orientation. In the mitogenomes of five *Ophiocordyceps* species reported, the number of ORFs was also different, e.g., the *H. thompsonii* mitogenome containing 3 ORFs [25], *H. rhossiliensis* mitogenome containing 5 ORFs [48], *H. minnesotensis* mitogenome containing 4 ORFs [24], as well as the *H. vermicola* mitogenome containing 3 ORFs [47].

To gain more insights into the variability of the mitochondrial gene among *Ophiocordyceps* species, the genomic arrangements of *O. pingbianensis* with other five mitogenomes in *Ophiocordyceps* were analyzed comparatively. The relative positions of most PCGs and most tRNAs of the six *Ophiocordyceps* species were relatively conservative, except for several tRNAs and PCGs distributed in different positions, which resulted from the variability of the relative gene order of these mitogenomes.

As revealed from genomic synteny, the six *Ophiocordyceps* mitogenomes fell into 4–5 homologous regions. Homologous region B only existed in the mitogenome of *O. pingbianensis* and *H. minnesotensis*, however, it was lacking in *H. thompsonii*, *H. vermicola*, *H. rhossiliensis* and *O. sinensis* mitogenomes. The relevant orders of four homologous regions (i.e., A, C, D and E) were conserved across the six *Ophiocordyceps* mitogenomes and homologous regions B were distributed in different positions among the mitogenomes; as a result, the diversity of gene order was generated among six mitogenomes. Gaining insights into gene arrangements, component and genomic synteny of *Ophiocordyceps* species might help assemble mitogenomes and trace the evolutionary history of other *Ophiocordycipitaceae* species in future research.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/life11070686/s1>, Table S1: Specimen information and GenBank accession numbers for sequences used in this study. Table S2: Specimen information and GenBank accession numbers for mitogenomes used in this study. Table S3: General features in the mitogenome of *Ophiocordyceps pingbianensis*. Table S4: Gene component of the mitogenome from *Ophiocordyceps pingbianensis*. Table S5: Codon usage of protein-coding genes in the mitogenome from *Ophiocordyceps pingbianensis*. Table S6: tRNAs in the mitogenome from *Ophiocordyceps pingbianensis*.

**Author Contributions:** Y.W. collected the specimen; H.Y., Y.W. and S.C. designed the experiments; S.C. and Y.W. conducted the experiments; S.C., Y.W. and K.Z. performed studies of morphology, mitogenomics and phylogeny; S.C. and Y.W. wrote the manuscript and H.Y. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Natural Science Foundation of China [31870017, 31760011], and the Department of Science and Technology of Yunnan Province [2018FY001(-006), 2018IA075].

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors wish to thank Qi Fan and Zhihong Xu for isolating and depositing strains of *O. puluogensis*.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Sung, G.H.; Hywel-Jones, N.L.; Sung, J.M.; Luangsa-Ard, J.J.; Shrestha, B.; Spatafora, J.W. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud. Mycol.* **2007**, *57*, 5–59. [[CrossRef](#)]
2. Petch, T. Notes on entomogenous fungi. *Trans. Brit. Mycol. Soc.* **1931**, *16*, 55–75. [[CrossRef](#)]
3. Shrestha, B.; Zhang, W.M.; Zhang, Y.J.; Liu, X.Z. What is the Chinese caterpillar fungus *Ophiocordyceps sinensis* (Ophiocordycipitaceae)? *Mycology* **2010**, *1*, 228–236. [[CrossRef](#)]
4. Kobayasi, Y. The genus *Cordyceps* and its allies. Science reports of the Tokyo Bunrika Daigaku. *Sci. Rep. Tokyo Bun. Daigaku* **1941**, *5*, 53–260.
5. Kobayasi, Y. Keys to the taxa of the genera *Cordyceps* and *Torrubiella*. *Trans. Mycol. Soc. Jpn.* **1982**, *23*, 329–364.
6. Mains, E.B. North American entomogenous species of *Cordyceps*. *Mycologia* **1958**, *50*, 169–222. [[CrossRef](#)]
7. Sanjuan, T.I.; Fanco-Molano, A.E.; Kepler, R.M.; Spatafora, J.W.; Tabima, J.; Vasco-palacios, A.M.; Restrepo, S. Five new species of entomopathogenic fungi from the Amazon and evolution of neotropical *Ophiocordyceps*. *Fungal Biol.* **2015**, *119*, 901–916. [[CrossRef](#)]
8. Spatafora, J.W.; Quandt, C.A.; Kepler, R.M.; Sung, G.H.; Shrestha, B.; Hywel-Jones, N.L.; Luangsa-ard, J.J. New 1F1N species combinations in Ophiocordycipitaceae (Hypocreales). *IMA Fungus* **2015**, *6*, 357–362. [[CrossRef](#)]

9. Araújo, J.P.M.; Evans, H.C.; Kepler, R.M.; Hughes, D.P. Zombie-ant fungi across continents: 15 new species and new combinations within *Ophiocordyceps*. I. Myrmecophilous hirsutelloid species. *Stud. Mycol.* **2018**, *90*, 119–160. [[CrossRef](#)]
10. Khonsanit, A.; Luangsa-ard, J.J.; Thanakitpipattana, D.; Kobmoo, N.; Piasai, O. Cryptic species within *Ophiocordyceps myrmecophila* complex on formicine ants from Thailand. *Mycol. Prog.* **2018**, *18*, 147–161. [[CrossRef](#)]
11. Luangsa-ard, J.J.; Tسانathai, K.; Thanakitpipattana, D.; Khonsanit, A.; Stadler, M. Novel and interesting *Ophiocordyceps* spp. (Ophiocordycipitaceae, Hypocreales) with superficial perithecia from Thailand. *Stud. Mycol.* **2018**, *89*, 125–142. [[CrossRef](#)] [[PubMed](#)]
12. Wijayawardene, N.N.; Hyde, K.D.; Lumbsch, H.T.; Liu, J.K.; Maharachchikumbura, S.S.; Ekanayaka, A.H.; Tian, Q.; Phookamsak, R. Outline of Ascomycota 2017. *Fungal Divers.* **2018**, *88*, 167–263. [[CrossRef](#)]
13. Tسانathai, K.; Noisripoom, W.; Chaitika, T.; Khonsanit, A.; Hasin, S.; Luangsa-Ard, J.J. Phylogenetic and morphological classification of *Ophiocordyceps* species on termites from Thailand. *MycologyKeys* **2019**, *56*, 101–129. [[CrossRef](#)] [[PubMed](#)]
14. Quandt, C.A.; Kepler, R.M.; Gams, W.; Araújo, J.P.; Ban, S.; Evans, H.C.; Luangsa-Ard, J.J. Phylogenetic-based nomenclatural proposals for Ophiocordycipitaceae (Hypocreales) with new combinations in *Tolyocladium*. *IMA Fungus* **2014**, *5*, 121–134. [[CrossRef](#)]
15. Ban, S.; Sakane, T.; Nakagiri, A. Three new species of *Ophiocordyceps* and overview of anamorph types in the genus and the family Ophiocordycepsaceae. *Mycol. Prog.* **2015**, *14*, 1017. [[CrossRef](#)]
16. Hodge, K.T. Revisionary Studies in *Hirsutella* (Anamorphic Hyphomycetes: Clavicipitaceae). Ph.D. Thesis, Cornell University, New York, NY, USA, 1998.
17. Simmons, D.R.; Kepler, R.M.; Rehner, S.A.; Groden, E. Phylogeny of *Hirsutella* species (Ophiocordycipitaceae) from the USA: Remediating the paucity of *Hirsutella* sequence data. *IMA Fungus* **2015**, *6*, 345–356. [[CrossRef](#)]
18. Wang, Y.B.; Nguyen, T.T.; Dai, Y.D.; Yu, H.; Wu, C.K. Molecular phylogeny and morphology of *Ophiocordyceps unituberculata* sp. nov. (Ophiocordycipitaceae), a pathogen of caterpillars (Noctuidae, Lepidoptera) from Yunnan, China. *Mycol. Prog.* **2018**, *17*, 745–753. [[CrossRef](#)]
19. Williams, S.T.; Foster, P.G.; Littlewood, D.T. The complete mitochondrial genome of a turbinid vetigastropod from MiSeq Illumina sequencing of genomic DNA and steps towards a resolved gastropod phylogeny. *Gene* **2014**, *533*, 38–47. [[CrossRef](#)]
20. Botero-Castro, F.; Tilak, M.K.; Justy, F.; Catzeflis, F.; Delsuc, F.; Douzery, E.J. Next generation sequencing and phylogenetic signal of complete mitochondrial genomes for resolving the evolutionary history of leaf-nosed bats (Phyllostomidae). *Mol. Phylogenet. Evol.* **2013**, *69*, 728–739. [[CrossRef](#)]
21. James, T.Y.; Pelin, A.; Bonen, L.; Ahrendt, S.; Sain, D.; Corradi, N.; Stajich, J.E. Shared signatures of parasitism and phylogenomics unite the Cryptomycota and Microsporidia. *Curr. Biol.* **2013**, *23*, 1548–1553. [[CrossRef](#)]
22. Liu, W.; Cai, Y.L.; Zhang, Q.Q.; Chen, L.F.; Shu, F.; Ma, X.L.; Bian, Y.B. The mitochondrial genome of *Morchella importuna* (272.2 kb) is the largest among fungi and contains numerous introns, mitochondrial non-conserved open reading frames and repetitive sequences. *Int. J. Biol. Macromol.* **2019**, *143*, 373–381. [[CrossRef](#)]
23. Kang, X.; Hu, L.; Shen, P.; Li, R.; Liu, D. SMRT sequencing revealed mitogenome characteristics and mitogenome-wide DNA modification pattern in *Ophiocordyceps sinensis*. *Front. Microbiol.* **2017**, *8*, 1422. [[CrossRef](#)]
24. Zhang, Y.J.; Zhang, S.; Liu, X. The complete mitochondrial genome of the nematode endoparasitic fungus *Hirsutella minnesotensis*. *Mitochondrial DNA Part A* **2016**, *27*, 2693–2694. [[CrossRef](#)] [[PubMed](#)]
25. Zhang, Y.J.; Wang, L.; Zhang, S.; Li, J.H. Mitochondrial genome, comparative analysis and evolutionary insights into the entomopathogenic fungus *Hirsutella thompsonii*. *Environ. Microbiol.* **2018**, *20*, 3393–3405. [[CrossRef](#)]
26. Wang, Y.B.; Wang, Y.; Fan, Q.; Duan, D.E.; Zhang, G.D.; Dai, R.Q.; Dai, Y.D.; Zeng, W.B.; Chen, Z.H.; Li, D.D.; et al. Multigene phylogeny of the family Cordycipitaceae (Hypocreales): New taxa and the new systematic position of the Chinese cordycipitoid fungus *Paecilomyces hepiali*. *Fungal Divers.* **2020**, *103*, 1–46. [[CrossRef](#)]
27. Liu, X.Z.; Chen, S.Y. Screening isolates of *Hirsutella* species for biocontrol of *Heterodera glycines*. *Biocontrol. Sci. Tech.* **2001**, *11*, 151–160. [[CrossRef](#)]
28. Wang, Y.B.; Yu, H.; Dai, Y.D.; Wu, C.K.; Zeng, W.B.; Yuan, F.; Liang, Z.Q. *Polycephalomyces agaricus*, a new hyperparasite of *Ophiocordyceps* sp. infecting melolonthid larvae in southwestern China. *Mycol. Prog.* **2015**, *14*, 70. [[CrossRef](#)]
29. Vilgalys, R.; Hester, M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* **1990**, *172*, 4238–4246. [[CrossRef](#)]
30. Rehner, S.A.; Samuels, G.J. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycol. Res.* **1994**, *98*, 625–634. [[CrossRef](#)]
31. Bischoff, J.F.; Rehner, S.A.; Humber, R.A. *Metarhizium frigidum* sp. nov.: A cryptic species of *M. anisopliae* and a member of the *M. flavoviride* Complex. *Mycologia* **2006**, *98*, 737–745. [[CrossRef](#)]
32. White, T.J.; Bruns, T.D.; Lee, S.B.; Taylor, J.W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *Pcr Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: New York, NY, USA, 1990; pp. 315–322.
33. Wang, Y.B.; Yu, H.; Dai, Y.D.; Chen, Z.H.; Zeng, W.B.; Yuan, F.; Liang, Z.Q. *Polycephalomyces yunnanensis* (Hypocreales), a new species of *Polycephalomyces* parasitizing *Ophiocordyceps nutans* and stink bugs (hemipteran adults). *Phytotaxa* **2015**, *208*, 34–44. [[CrossRef](#)]

34. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.L.; Pham, S.; Pribelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [[CrossRef](#)]
35. Valach, M.; Burger, G.; Gray, M.W.; Lang, B.F. Widespread occurrence of organelle genome-encoded 5S rRNAs including permuted molecules. *Nucleic Acids Res.* **2014**, *42*, 13764–13777. [[CrossRef](#)]
36. Lohse, M.; Drechsel, O.; Bock, R. Organellargenomedraw (ogdraw): A tool for the easy generation of high-quality custom graphical maps of plastid and mitochondrial genomes. *Curr. Genet.* **2007**, *52*, 267–274. [[CrossRef](#)]
37. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948. [[CrossRef](#)]
38. Tamura, K.; Stecher, G.; Peterson, D.; Filipowski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [[CrossRef](#)]
39. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [[CrossRef](#)]
40. Stamatakis, A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **2006**, *22*, 2688–2690. [[CrossRef](#)] [[PubMed](#)]
41. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. **2003**, *19*, 1572–1574. [[CrossRef](#)]
42. Brady, S. Notes on *Hirsutella aphidis*. *Trans. Br. Mycol. Soc.* **1985**, *85*, 752–756. [[CrossRef](#)]
43. Zou, X.; Liu, A.Y.; Liang, Z.Q.; Han, Y.N.; Yang, M.F. *Hirsutella liboensis*, a new entomopathogenic species affecting Cossidae (Lepidoptera) in China. *Mycotaxon* **2010**, *111*, 34–44. [[CrossRef](#)]
44. Minter, D.W.; Brady, B.L. Mononematous species of *Hirsutella*. *Trans. Br. Mycol. Soc.* **1980**, *74*, 271–282. [[CrossRef](#)]
45. Aoki, K.; Nakasato, Y.; Fujimoto, I.; Suzuki, K. Studies on the new fungous parasites of silkworms, Bombyx Mori L. IV. *Hirsutella satumaensis* Aoki sp. nov. *Bull. Seric. Exp. Stn.* **1957**, *14*, 471–486.
46. Zou, X.; Zhou, Y.M.; Liang, Z.Q.; Xu, F.L. A new species of the genus *Hirsutella* with helical twist neck of phialides parasitized on Tortricidae. *Mycosystema* **2016**, *53*, 807–813.
47. Xiang, M.C.; Yang, E.C.; Xiao, Q.M.; Liu, X.Z.; Chen, S.Y. *Hirsutella vermicola* sp. nov., a new species parasitizing bacteria-feeding nematodes. *Fungal Divers.* **2006**, *19*, 217–234. [[CrossRef](#)]
48. Wang, N.N.; Zhang, Y.J.; Hussain, M.; Li, K.; Xiang, M.H.; Liu, X.Z. The mitochondrial genome of the nematode endoparasitic fungus *Hirsutella rhossiliensis*. *Mitochondrial DNA Part B* **2016**, *27*, 2693–2694. [[CrossRef](#)]