Phylogeny and Systematics of the Genus *Tolypocladium* (Ophiocordycipitaceae, Hypocreales)

Quan-Ying Dong 1,2, Yao Wang 1,*  Zhi-Qin Wang 1,2, Yan-Fang Liu 2 and Hong Yu 2,*

1 Yunnan Herbal Laboratory, College of Ecology and Environmental Sciences, Yunnan University, Kunming 650504, China
2 The International Joint Research Center for Sustainable Utilization of Cordyceps Bioresources in China and Southeast Asia, Yunnan University, Kunming 650504, China
* Correspondence: wangyao1@aliyun.com (Y.W.); hongyu@ynu.edu.cn (H.Y.); Tel.: +86-13700676633 (H.Y.)

Abstract: The taxonomy and phylogeny of the genus *Tolypocladium* are herein revised based on the most comprehensive dataset to date. Two species-level phylogenies of *Tolypocladium* were constructed: a single-gene phylogeny (ITS) of 35 accepted species and a multigene phylogeny (nrSSU, nrLSU, tef-1a, rpb1, and rpb2) of 27 accepted species. Three new species, *Tolypocladium pseudoalbum* sp. nov., *Tolypocladium subparadoxum* sp. nov., and *Tolypocladium yunnanense* sp. nov., are described in the present study. The genetic divergences of four markers (ITS, tef-1a, rpb1 and rpb2) among *Tolypocladium* species are also reported. The results indicated that species of *Tolypocladium* were best delimitated by rpb1 sequence data, followed by the sequence data for the rpb2, tef-1a, and ITS provided regions. Finally, a key to the 48 accepted species of *Tolypocladium* worldwide is provided.

Keywords: micromorphology; phylogenetic analyses; taxonomy; three new taxa

1. Introduction

*Tolypocladium* was originally described as an anamorph genus by Gams in 1971 to accommodate three species collected from soil: *T. cylindrosporum* W. Gams, *T. geodes* W. Gams, and *T. inflatum* W. Gams [1]. Subsequently, the species *T. lignicola* G.L. Barron, *T. parasiticum* G.L. Barron, and *T. trigonomosporum* G.L. Barron, all of which were isolated from bdelloid rotifers, were added to this genus [2–4]. Bissett described *T. nubicola* and *T. tundrense* from soil in 1983 [5] and reassigned three species to *Tolypocladium*: *T. balanoides* (basionym: *Cephalosporium balanoide*), *T. microsporum* (basionym: *Verticillium microsporum*) and *T. niveum* (basionym: *Pachybasium niveum*). Additionally, Bissett [5] noted that the morphological characteristics of *T. niveum* were similar to those of *T. inflatum*. Because *T. niveum* preceedes *T. inflatum*, Bissett proposed that *T. inflatum* be synonymized with *T. niveum* [5]. However, Dreyfuss observed that *T. inflatum* produces cyclosporine and is the type species of the genus *Tolypocladium*. The name *T. inflatum* is also commonly accepted [6]. Therefore, Dreyfuss rejected the synonymization of *T. inflatum* with *T. niveum* [6]. The genus *Tolypocladium* is morphologically characterized by sparingly branched conidiophores, swollen phialides, and one-celled conidia borne in slimy heads. Approximately 20 species have been included in the *Tolypocladium* based on morphological characteristics.

The taxonomy of *Tolypocladium* has been discussed extensively for decades. *Cordyceps* sensu lato was recently reclassified into three families (Clavicipitaceae sensu stricto, Cordycepiaceae, and Ophiocordycipitaceae) and four genera (*Cordyceps* s. str., *Elaphocordycites*, *Metacordyceps*, and *Ophiocordyceps*) based on multigene phylogeny [7]. Molecular phylogenetic analyses suggested that *Tolypocladium* species fall within the Ophiocordycipitaceae [7,8]. The genus *Elaphocordycites* Sung and Spatafora 2007 was proposed for 23 species of the *Cordyceps* Fr. (1818: 316); these species parasitize the fungal genus *Elaphomyces* and some species of arthropods (e.g., cicada nymphs and beetle larvae) [7]. The *Elaphocordycites* species within the Ophiocordycipitaceae form a clade sister to those of the genus *Ophiocordyceps*.
Gams established the *Chaunopycnis* to accommodate *C. alba*, which morphologically resembles *Tolypocladium* species in its conidiogenesis [9]. With the end of dual nomenclature for fungi, the generic name *Tolypocladium* was chosen over *Elaphocordyceps* and *Chaunopycnis* as *Tolypocladium* is the oldest and most commonly used name [8]. *Chaunopycnis* was integrated into the genus *Tolypocladium*. Accordingly, *C. alba*, *C. ovalispora*, and *C. pustulata* were renamed *T. album*, *T. ovalisporum*, and *T. pustulatum*, respectively [8].

At present, 53 *Tolypocladium* records, including 5 varieties, are listed in the *Index Fungorum* ([www.indexfungorum.org](http://www.indexfungorum.org), accessed on 28 August 2022). *Tolypocladium balanoides*, which was reassigned to *Drechmeria* (as *Drechmeria balanoides*), and *Tolypocladium parasiticum*, which was reassigned to *Metapochonia* (as *Tolypocladium parasiticum*), should be excluded from the *Tolypocladium*. However, some of these records are doubtful, because the original identifications were presumptive based on host associations or based on the morphology of only one or two ascospore stages of the asexual or sexual morph. For 16 species, no molecular data are available in the GenBank database [10]. *Tolypocladium* species have a cosmopolitan distribution and a broad host range that includes bdelloid rotifers, mosquito larvae, nematodes, fireflies, beetles, cicada nymphs, batmoth larvae, macrocystic fungi, *Ophiocordyceps sinensis*, and even plants (as endophytes) [2,3,11–19].

*Tolypocladium* species have been widely studied due to their importance in the medicinal domain. These species can produce cyclosporine A, tolypoalbin, tolypin, cyclosporine D hydroperoxide, cylindromicin, and tolyprolinol [20,21], all of which have significant antitumor, anti-inflammatory, antifungal, and/or antiparasitic properties [22]. Cyclosporine A, which is naturally isolated from *T. inflatum*, is widely used in autoimmune disease treatment and to prevent allograft rejection [23–25]. Tolyoalbin is a peptide mixture and a tetrameric acid produced by *T. album* [26]. Tolypin is also a peptide mixture [27]. Like kojic acid, cylindromicin is a significant bioactive inhibitor of tyrosinase [28]. Tolyprolinol, a dipeptide produced by *Tolypocladium* sp. FKI-7981, contains a rare moiety prolinol and was the first natural product isolated from *Tolypocladium* species. Tolyprolinol exhibits moderate antimalarial activity without cytotoxicity or any other antimicrobial properties [29].

Recent investigations and phylogenetic analyses have ascribed many new taxa to *Tolypocladium*. Therefore, the diversity of *Tolypocladium* may be underestimated. In the present study, we aimed first to investigate and document the worldwide diversity of *Tolypocladium* fungi using our current collection of specimens and data collected over the last several years. We used comprehensive morphological and molecular phylogenetic reconstructions to identify and reevaluate our specimens. Based on these reconstructions, we herein describe and illustrate three new taxa. We then clarify the phylogenetic affinities of these new taxa using rDNA sequence analyses.

### 2. Materials and Methods

#### 2.1. Sampling

*Tolypocladium* species were collected in Kunming, Pu’er, Yunnan, China. Voucher specimens and the corresponding isolated strains were deposited in the Yunnan Herbal Herbarium (YHH) and the Yunnan Fungal Culture Collection (YFCC), respectively, of Yunnan University, Kunming, China.

*Tolypocladium* strains were isolated from soil samples, as described in our previous publication [30]. In brief, 2 g of soil was added to a flask containing 20 mL of sterilized water and glass beads. The suspension was then shaken for 10 min and diluted 100 times. Finally, 200 µL of diluted soil suspension was spread on petri dishes containing solidified onion garlic agar (OGA: 1 L of distilled water, 20 g of grated garlic, and 20 g of onion were boiled together for 1 h; the boiled biomass was filtered and 2% agar was added to the filtrate). Czapek yeast extract agar (CYA; Advanced Technology and Industrial Co., Ltd., Hong Kong, China) and potato dextrose agar (PDA; Difco, USA) were used. Rose bengal (50 mg/L) and kanamycin (100 mg/L) were added to all media. Conidia grown on insect cadavers were transferred to PDA plates and cultured at 22 °C. The filamentous fungal colonies isolated from the culture were transferred to fresh PDA media. The purified fungal
strains were maintained at 22°C in a culture room or transferred to PDA slants and stored at 4°C.

2.2. Morphological Studies

Morphological studies were performed as described in our previous study [31]. Micromorphological characteristics, such as phialides and conidia, were studied by picking and mounting cultures on glass slides. The sizes and shapes of the microcharacteristics were determined using an Olympus CX40 and BX53 (Olympus Corporation, Tokyo, Japan). Individual length and width measurements were taken for 20–30 replicates, including the absolute minima and maxima. The morphological characteristics were described based on the digital images and the measurement dataset.

2.3. Molecular Studies

2.3.1. DNA Extraction and PCR Amplification

Total DNA was extracted from the fungal mycelia on PDA plates or from herbarium materials using the modified CTAB procedure [32]. The primer pair nrSSU-CoF and nrSSU-CoR [33] was used to amplify nrSSU, the primer pair LR5 and LR0R [34,35] was used to amplify nrLSU, and the primer pair EF1α-EF and EF1α-ER [7,36] was used to amplify the translation elongation factor 1α (tef-1α). The primer pair RPB1-5′F and RPB1-5′R and the primer pair RPB2-5′F and RPB2-5′R [7,36] were used to amplify the largest and second-largest subunits of RNA polymerase II (rpb1 and rpb2), respectively. The ITS fragment was amplified using the primer pair ITS5 and ITS4 [37].

The matrix for the polymerase chain reaction (PCR) was comprised of 2.5 µL PCR 10× buffer (2 mmol/L Mg²⁺) (Transgen Biotech, Beijing, China), 1 µL forward primer (10 µmol/L), 1 µL reverse primer (10 µmol/L), 0.25 µL Taq DNA polymerase (Transgen Biotech, Beijing, China), 2 µL dNTP (2.5 mmol/L), 1 µL DNA template (500 ng/µL), and 17.25 µL sterile ddH₂O. Amplification reactions were performed in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA). The PCR cycling conditions for the amplification of nrSSU were as follows: 95°C for 4 min; eight cycles of 94°C for 50 s, 56°C for 50 s, and 72°C for 2 min, with the annealing temperature decreasing 0.5°C/cycle; 25 cycles of 94°C for 50 s, 52°C for 50 s, and 72°C for 2 min; and 72°C for 10 min. The nucleotide sequences of ITS, nrLSU, tef-1α, rpb1, and rpb2 were amplified using the following cycling conditions: 95°C for 4 min; eight cycles of 94°C for 50 s, 56°C for 50 s, and 72°C for 70 s, with the annealing temperature decreasing 0.5°C/cycle; 25 cycles of 94°C for 50 s, 52°C for 50 s, and 72°C for 70 s; and 72°C for 10 min. PCR products were purified using a gel extraction and PCR purification combo kit (Beijing Genomics Institute, Shenzhen, China) and sequenced on an automatic sequence analyzer (BGI Co., Ltd., Shenzhen, China) using the amplification primers.

2.3.2. DNA Sequence Alignments

To investigate the placement of our samples within Tolypocladium, the nucleotide sequences of ITS, nrSSU, nrLSU, tef-1α, rpb1, and rpb2 were compared with sequences from representative Tolypocladium species downloaded from GenBank (Table 1, Figures 1 and 2). Individual gene sequence datasets (ITS, nrSSU, nrLSU, tef-1α, rpb1, and rpb2) were aligned and manually checked using Bioedit v7.0.9 [38]. To identify possible phylogenetic conflicts among the datasets, the partition homogeneity (PH) test was performed with 1000 randomized replicates of heuristic searches with simple sequence addition in PAUP* 4.0a166 (http://paup.phylosolutions.com, accessed on 28 August 2022) [39]. The results showed that the phylogenetic signals from the five gene markers were in conflict.
Figure 1. Maximum-likelihood tree illustrating the phylogeny of *Tolypocladium* based on the combined dataset of nrSSU, nrLSU, tef-1α, rpb1 and rpb2 sequences. *Polycephalomyces formosus* ARSEF 1424 and *Polycephalomyces sinensis* CN 80-2 were used as outgroups. The maximum-likelihood bootstrap values (≥50) and Bayesian posterior probability values (≥0.50) are indicated above the branches. Isolates in bold type are those analyzed in this study.
Figure 2. Maximum parsimony, Bayesian analysis, and RAxML tree illustrating the phylogeny of *Tolypocladium* derived from ITS sequences. Statistical support values (MP bootstrap/Bayesian posterior probability/ML bootstrap ≥ 70%) are shown at the nodes. The indistinguishable species are in red and the isolates analyzed in this study are in bold.

2.3.3. Phylogenetic Analyses

Phylogenetic analyses were based on a concatenated five-gene dataset and the ITS sequences alone. *nrSSU*, *nlLSU*, *tef1α*, *rpb1*, and *rpb2*, and ITS sequences were retrieved from GenBank, and combined with those generated in this study. Taxon information and GenBank accession numbers are given in Table 1. Sequences were aligned using Clustal X2.0 and MEGA v6.06 [40,41]. Group I introns in the *nrSSU* sequences of some species were excluded from the phylogenetic analyses, and gaps were treated as missing data. After
alignment of the five genes individually, the alignments were concatenated. A partition homogeneity test was conducted in PAUP* 4.0a166 [39], and the results indicated that there were no conflicts among the data partitions. PartitionFinder V1.1.1 identified eleven data partitions: nine corresponding to the three codon positions in each of the protein-coding genes (\textit{tef-1a}, \textit{rpb1}, and \textit{rpb2}) and one each for \textit{nrLSU} and \textit{nrSSU} [42,43]. The results showed that the phylogenetic signals of the five genes were congruent ($p = 0.02$).

Maximum likelihood (ML) phylogenetic analyses were conducted using RaxML 7.0.3 [44] with the recommended partition parameters and 1000 rapid bootstrap replicates. Bayesian posterior probabilities (BP) were estimated with the same partition parameters using MrBayes v3.1.2 [45]. Bayesian inference (BI) analysis ran in MrBayes v3.1.2 for 5 million generations. Maximum parsimony (MP) analysis of the ITS dataset was performed using PAUP v. 4.0a166 [39], adopting the random addition of sequences model (10 replications), with gaps treated as missing data. A bootstrap (MPBS) analysis was performed using the maximum parsimony criterion in 1000 replications.

The following taxa were included in the five-gene concatenated dataset: \textit{Drechmeria W. Gams and H.-B. Jansson}, \textit{Harposporium Lohde}, \textit{Ophiocordyceps Petch}, \textit{Purpureocillium Luangsra-Ard}, \textit{Hywel-Jones}, \textit{Houbraken} and \textit{Samson}, and \textit{Tolypocladium}. Two species of \textit{Polycephalomyces Kobayasi} were used as outgroups. ITS analysis was performed on \textit{Tolypocladium} taxa only. Phylogenetic trees were visualized with FigTree v1.4.0 [46], edited in Microsoft PowerPoint, saved in PDF format, and converted to JPG format using Adobe Illustrator CS6 (Adobe Systems Inc., San Jose, USA). The finalized alignments and trees were submitted to TreeBASE (multigene submission ID 29808).

We calculated a phylogenetic distance matrix for the markers ITS, \textit{tef-1a}, \textit{rpb1}, and \textit{rpb2} to assess the species boundaries of the 10 \textit{Tolypocladium} species (Supplementary Tables S1–S4), because the sequence data were complete for these four loci. The paired distances among the 10 \textit{Tolypocladium} lineages were measured using the Kimura two-parameter model in MEGA v6.06 [41].
Table 1. Specimen information and GenBank accession numbers of sequences used in this study.

| Taxon                                | Voucher Information | GenBank Accession Numbers | Reference |
|--------------------------------------|---------------------|---------------------------|-----------|
| Drechmeria balanoides                | CBS 250.82          | AF339588                  | [47,48]   |
| Drechmeria campanulata               | IMI 366051          | AF339592                  | [47]      |
| Drechmeria coniospora                | ARSEF 6992          | AF339572                  | [49]      |
| Drechmeria gunnii                    | OSC 76404           | AF339522                  | [48,47]   |
| Drechmeria panacis                   | CBS 142798          | AF339589                  | [50]      |
| Drechmeria sinensis                  | CBS 567.95          | AF339594                  | [47]      |
| Drechmeria sphaerospora              | CBS 522.80          | AF339590                  | [47]      |
| Drechmeria zeospora                  | CBS 335.80          | AF339589                  | [47,47]   |
| Harposporium anguillulae             | ARSEF 5407          | MF588890                  | [51]      |
| Harposporium anguillulae             | ARSEF 5993          | MF588891                  | [51]      |
| Harposporium harposporiferum         | ARSEF 5727          | AF339569                  | [47,48]   |
| Harposporium helicoides              | ARSEF 5334          | AF339577                  | [47]      |
| Hirsutella citiformis                | ARSEF 1446          | KM652065                  | [52]      |
| Hirsutella crypoclerotrichum         | ARSEF 4517          | KM652066                  | [52]      |
| Hirsutella fusiformis                | ARSEF 5474          | KM652067                  | [52]      |
| Hirsutella guyana                    | ARSEF 878           | KM652068                  | [52]      |
| Hirsutella illustri                 | ARSEF 5539          | KM652069                  | [52]      |
| Hirsutella lecanicola                | ARSEF 8888          | KM652071                  | [52]      |
| Hirsutella minnesota                  | ARSEF 3608          | JPU100000376              | [53]      |
| Hirsutella nactrix                   | ARSEF 5549          | KM652073                  | [52]      |
| Hirsutella nodulosa                  | ARSEF 5473          | KM652074                  | [52]      |
| Hirsutella nautae                    | ARSEF 1369          | KM652076                  | [52]      |
| Hirsutella rhosilensis               | ARSEF 3747          | KM652080                  | [52]      |
| Hirsutella satumane                 | ARSEF 996           | KM652082                  | [52]      |
| Hirsutella strigose                  | ARSEF 2197          | KM652085                  | [52]      |
| Hirsutella subulata                  | ARSEF 2227          | KM652086                  | [52]      |
| Hirsutella thompsonii                | ARSEF 1037          | KM652092                  | [52]      |
| Hirsutella versicolor                | ARSEF 1037          | KM652102                  | [52]      |
| Hirsutella accicularis               | OSC 110987          | EF468950                  | [52]      |
| Hirsutella accicularis               | OSC 110988          | EF468951                  | [52]      |
| Hirsutella agrioides                 | ARSEF 5692          | DQ522540                  | [48]      |
| Hirsutella amazonica                 | HUA 16143           | KJ197562                  | [48]      |
| Hirsutella appendiculata             | NBRC 10696          | JN941728                  | [56,57]   |
| Hirsutella arborescens               | NBRC 105891         | AB968386                  | [56]      |
| Hirsutella bispora                   | ERS112307           | FKNF01000183              | [58]      |
| Hirsutella blattarioides             | HUA 16108           | KJ197558                  | [55]      |
| Hirsutella brunneaeigina             | TBRC 8093           | MF614654                  | [59]      |
| Hirsutella brunneipunctata           | OSC 128576          | DQ522542                  | [48]      |
Table 1. Cont.

| Taxon | Voucher Information | GenBank Accession Number | Reference |
|-------|---------------------|--------------------------|-----------|
| **Ophiocordyceps cf. acicularis** | OSC 128580 | DQ522543 | DQ518757 | DQ522326 | DQ522371 | DQ522423 | [48] |
| **Ophiocordyceps cininalis** | GADM 17327 | KF226253 | KF226254 | KF226256 | KF226255 | - | [60] |
| **Ophiocordyceps entomorrhiza** | KEW 53484 | E4F68894 | E4F68890 | E4F68749 | E4F68857 | E4F68911 | [7] |
| **Ophiocordyceps geometridicola** | TBRC 8095T | - | MF14648 | MF161463 | MF161466 | MF161479 | [59] |
| **Ophiocordyceps gracilis** | EFCC 8572 | E4F68956 | E4F68881 | E4F68751 | E4F68859 | E4F68912 | [7] |
| **Ophiocordyceps heteropoda** | NBRC 100644 | JN941718 | JN941423 | AB966596 | JN992452 | AB968557 | [56, 57] |
| **Ophiocordyceps kniphofioides** | HUA 186148 | KC610790 | KC610739 | KC610767 | KC610717 [5] |
| **Ophiocordyceps lanpingensis** | YHOS0705 | KC417458 | KC417460 | KC417462 | KC417464 | KC456333 | [61] |
| **Ophiocordyceps macroacicularis** | NBRC 100685 | AB968388 | T | AB968574 | - | AB968536 | [56] |
| **Ophiocordyceps multiperitheciata** | BCC 69008 | MF614648 | MF614632 | MF614663 | MF614679 | - | [59] |
| **Ophiocordyceps nigrella** | EFCC 9247 | E4F68963 | E4F68818 | E4F68758 | E4F68866 | E4F68920 | [7] |
| **Ophiocordyceps nooreniae** | BRIP 55363 | T | KX673811 | KX673810 | KX673812 | - | KX673809 | [62] |
| **Ophiocordyceps pseudoacicularis** | TBRC 8102 | MF614646 | MF614630 | MF614661 | MF614677 | - | [59] |
| **Ophiocordyceps pruinosa** | NHJ 12094 | EU369016 | EU369041 | EU369076 | EU369084 | - | [63] |
| **Ophiocordyceps ravenellii** | OSC 110995 | DQ522550 | DQ518764 | DQ522334 | DQ522379 | DQ522430 | [48] |
| **Ophiocordyceps rhizoidea** | NHJ 12522 | E4F68970 | E4F68825 | E4F68764 | E4F68873 | E4F68923 | [7] |
| **Ophiocordyceps rubignosisperitheciata** | NBRC 106966 | JN941704 | JN941437 | AB968382 | JN992438 | AB968544 | [56, 57] |
| **Ophiocordyceps sinensis** | EFCC 7287 | EF468971 | EF468827 | EF468767 | EF468874 | - | [7] |
| **Ophiocordyceps spatulata** | BCC 86480T | - | MG831747 | MG831746 | MG831748 | MG831749 | [59] |
| **Ophiocordyceps stylopora** | OSC 110999 | E4F68982 | E4F68837 | E4F68777 | E4F68882 | E4F68931 | [7] |
| **Ophiocordyceps unilateralis** | OSC 128574 | DQ522554 | DQ518768 | DQ522339 | DQ522835 | DQ522436 | [48] |
| **Ophiocordyceps unilateralis** | VFCC HU1031T | KT923214 | KT923212 | KT923216 | KT923218 | KT923220 | [65] |
| **Ophiocordyceps variabilis** | ARSEF 5565 | DQ522555 | DQ518769 | DQ522340 | DQ522386 | DQ522437 | [48] |
| **Ophiocordyceps xuefengensis** | GZUH2012HN14T | MG31793 | - | MG31793 | MG31794 | MG31795 | [66] |
| **Pseudocordyceps formosus** | ARSEF 1424 | KF409615 | AY255944 | DQ118754 | DQ127245 | KF409671 | [43, 51, 67, 68] |
| **Pseudocordyceps sinensis** | CN 80-2 | HQ832887 | HQ832886 | HQ832890 | HQ832888 | HQ832889 | [69] |
| **Purpurocordyceps atypicolum** | CBS 744.73 | E4F68987 | E4F68841 | E4F68786 | E4F68892 | - | [7] |
| **Purpurocordyceps atypicolum** | OSC 15901 | KJ978991 | KJ978990 | KJ978991 | KJ978992 | - | [8] |
| **Purpurocordyceps lavendulum** | CBS 128677T | FR775489 | FR775516 | FR775512 | FR775538 | - | [70] |
| **Purpurocordyceps lilacinum** | CBS 284.36T | AV526475 | FR775484 | E4F68792 | E4F68898 | E4F68941 | [70, 71] |
| **Purpurocordyceps lilacinum** | NHJ 3582 | EU369096 | EU369033 | EU369014 | EU369053 | EU369074 | [63] |
| **Purpurocordyceps takamizusanense** | NHJ 3582 | EU369097 | EU369034 | EU369015 | EU369056 | EU369075 | [63] |
| **Tolypocladium amadum** | CBS 136895T | KF747314 | KF747134 | KF747099 | KF747214 | - | [72] |
| **Tolypocladium bacillisporum** | C23 | LC684522 | LC684525 | LC684528 | - | LC684525 | [13] |
| **Tolypocladium capitatum** | NBRC 100997 | JN941740 | JN941401 | AB968597 | JN992474 | AB968558 | [56, 57] |
| **Tolypocladium capitatum** | NBRC 106325 | JN941739 | JN941402 | AB968598 | JN992473 | AB968559 | [56, 57] |
| **Tolypocladium capitatum** | YFCC 881 | OP207711 | OP207731 | OP223145 | OP223123 | OP223133 | Present study |
| **Tolypocladium cucullae** | GZU A-77 | MW798785 | MW798787 | - | - | - | [73] |
| **Tolypocladium cucullae** | HKAS 55588 | MW798784 | MW798786 | - | - | - | [73] |
### Table 1. Cont.

| Taxon                        | Voucher Information   | GenBank Accession Number | Reference |
|------------------------------|-----------------------|--------------------------|-----------|
| Tolypocladium cyindrosporum  | ARSEF 2920<sup>T</sup> | MH871712                | [15,74]   |
| Tolypocladium cyindrosporum  | YFCC 1805001          | MK984565                | [11]      |
| Tolypocladium endophyticum   | MS337                 | KF747315                | [72]      |
| Tolypocladium endophyticum   | MX486                 | KF747321                | [72]      |
| Tolypocladium flavocinum     | BCC 66576             | MN337287                | [14]      |
| Tolypocladium flavocinum     | BCC 66580             | MN338497                | [14]      |
| Tolypocladium fractum        | OSC 110990            | DQ522545                | [48]      |
| Tolypocladium fumosum        | CBS H-22968<sup>T</sup>| KU985053                | [75]      |
| Tolypocladium geodes         | CBS 126054            | MH875520                | [74]      |
| Tolypocladium inegoense      | SU-15                 | DQ118741                | [51]      |
| Tolypocladium innotatum      | OSC 71235             | EF469077                | [7]       |
| Tolypocladium insusticatipum| HKAS 112152           | MW537718                | [12]      |
| Tolypocladium insusticatipum| HKAS 112153           | MW537719                | [12]      |
| Tolypocladium japonicum      | NBRC 9647             | OP207712                | Present study |
| Tolypocladium jezeense       | NBRC 106328           | OP207713                | Present study |
| Tolypocladium longisegmentum| OSC 110992            | EF468816                | [7]       |
| Tolypocladium nubicola       | CBS 568.84<sup>T</sup> | MH873478                | [74]      |
| Tolypocladium ophioglossoides| CBS 100239            | KJ878910                | [8]       |
| Tolypocladium ophioglossoides| NBRC 100998           | JN941375                | [56,57]   |
| Tolypocladium ophioglossoides| NBRC 106330           | JN941407                | [56,57]   |
| Tolypocladium paradoxum      | NBRC 100945           | JN941410                | [56,57]   |
| Tolypocladium paradoxum      | YFCC 882              | OP207714                | Present study |
| Tolypocladium pseudobulbium  | YFCC 875<sup>T</sup>  | OP207717                | Present study |
| Tolypocladium pseudobulbium  | YFCC 876              | OP207718                | Present study |
| Tolypocladium pseudoalbum    | MRL GB6597            | AF389190                | [18]      |
| Tolypocladium pseudoalbum    | MRL MF5368LR          | AF373282                | [18]      |
| Tolypocladium reniformisporum| YFCC 1805002<sup>T</sup> | MK984566                | [11]      |
| Tolypocladium sp.            | YFCC 201803           | MK984567                | [11]      |
| Tolypocladium subparadoxum   | NBRC 106958           | OP207715                | Present study |
| Tolypocladium subparadoxum   | YFCC 879<sup>T</sup>  | OP207716                | Present study |
| Tolypocladium tropical       | CBS 136897<sup>T</sup>| KF747318                | [72]      |
| Tolypocladium tropical       | MX338                 | KF747321                | [72]      |
| Tolypocladium tundrense      | CBS 569.84<sup>T</sup> | MH873479                | [74]      |
| Tolypocladium yunnanense     | YFCC 877<sup>T</sup>  | OP207719                | Present study |
| Tolypocladium yunnanense     | YFCC 878              | OP207720                | Present study |

**Boldface:** data generated in this study. **<sup>T</sup>** ex-type material.
3. Results

3.1. Sequence Alignment and Phylogenetic Analyses

ITS, nrSSU, nrLSU, tef-1α, rpb1, and rpb2 sequences were generated from ten living cultures (accession numbers are given in Table 1). The concatenated five-gene alignment of 113 taxa contained 5371 base pairs in total: nrSSU, 1488 bp; nrLSU, 987 bp; tef-1α, 998 bp; rpb1, 756 bp; and rpb2, 1142 bp. *Polycephalomyces formosus* ARSEF 1424 and *Polycephalomyces sinensis* CN 80-2 were used as the outgroup sequences for the five-gene phylogenetic analyses. Both BI and ML analyses recovered six well-supported clades corresponding to the *Ophiocordyceps* (ML bootstrap, BS = 85% and bayesian posterior probability, BP = 1), *Tolypocladium* (BS = 99%, BP = 1), *Purpureocillium* (BS = 97%, BP = 1), *Drechmeria* (BS = 97%, BP = 1), *Harposporium* (BS = 88%, BP = 1), and *Polycephalomyces* (BS = 100%, BP = 1) (Figure 1) within Ophiocordycipitaceae. Phylogenetically, the *Tolypocladium* clade is the closest to the *Ophiocordyceps* clade, and it is well supported in this and other published analyses [7,8]. According to the current data, relationships for species in the *Tolypocladium* clade show strong statistical support for internal branches. Most sexual species are located at the top of the *Tolypocladium* clade, and asexual species are located at the bottom of the *Tolypocladium* clade, except *T. subparadoxum* and *T. paradoxum*. Three new species (i.e., *Tolypocladium pseudoalbum* sp. nov., *Tolypocladium subparadoxum* sp. nov., and *Tolypocladium yunnanense* sp. nov.) were recognized in *Tolypocladium* (shown in boldface in Figure 1). *T. pseudoalbum* sp. nov. formed a clade with *T. pustulatum*, *T. tropicale*, *T. endophyticum*, *T. amazonense*, and *T. yunnanense* sp. nov. (Figure 1), while *T. subparadoxum* sp. nov. formed a well-supported clade with *Tolypocladium* sp. and *T. paradoxum* (Figure 1). *T. yunnanense* sp. nov. was close to five other species: *T. pustulatum*, *T. tropicale*, *T. endophyticum*, *T. amazonense*, and *T. pseudoalbum* sp. nov. (Figure 1).

The ITS dataset used for phylogenetic analyses comprised 769 base pairs of sequence data for 61 taxa. *Purpureocillium lilacinum* CBS 284.36 and *Purpureocillium lilacinum* NHJ 3497 were chosen as outgroup sequences. The three phylogenetic algorithms (BI, ML, and MP) recovered trees with similar topologies (Figure 2). The three new species described herein (i.e., *Tolypocladium pseudoalbum* sp. nov., *Tolypocladium subparadoxum* sp. nov., and *Tolypocladium yunnanense* sp. nov.) formed an independent lineage with *Tolypocladium* (Figure 2).

3.2. Genetic Distance Analyses

Comparisons of genetic divergence showed that (1) the minimum thresholds (p-distances) required to distinguish species within the *Tolypocladium* lineages were 0.026, 0.017, 0.013, and 0.008 for tef-1α, rpb1, rpb2, and ITS, respectively (Supplementary Tables S1–S4); and (2) the phylogenetic relationships within *Tolypocladium* were best resolved by the rpb1 sequence data, followed by those of rpb2, tef-1α, and ITS (Supplementary Tables S1–S4).

3.3. Taxonomy

*Tolypocladium* W. Gams, Persoonia 6(2): 185 (1971). emend. C. A. Quandt et al. IMA Fungus 5: 125 (2014).

**Synonyms:** *Chaunopycnis* W. Gams, Persoonia 11: 75 (1980).

*Elaphocordyceps* G. H. Sung and Spatafora, Stud. Mycol. 57: 36 (2007).

**Sexual morph:** Stromata are solitary or several, simple or branched. The stipe is tough, dark-brownish to greenish, cylindrical, and abruptly to enlarging in the fertile part. The fertile part is cylindrical to clavate. Perithecia are superficial, wholly or partially immersed, ordinal or oblique in arrangement. Ascii are cylindrical with a thickened ascus apex. Ascospores are usually cylindrical, multisepate, disarticulate into part spores, and are occasionally non-disarticulating. Part spores are cylindrical.

**Asexual morph:** *Tolypocladium*-like, *Chaunopycnis*-like, or *Verticillium*-like. Conidiophores typically are short and bear whorls of phialides. Phialides often have bent necks and are usually swollen at the base. Conidia are ellipsoidal, globose, or reniform, and aggregate in small heads at the tips of the phialides.
Figure 3. Morphology of *Tolypocladium pseudoalbum* (YFCC 875, ex-type living culture). (A, B) Culture characteristics on PDA medium incubated at 22 °C for 14 days; (C–I) phialides; (J) conidia; (K) chlamydospore. Scale bars: (A, B) = 10 mm; (C–H) = 20 μm; (I–K) = 10 μm.
**MycoBank:** MB 845430.

**Etymology:** Referring to the morphological resemblance of this species to *Tolypocladium album*, despite its phylogenetic dissimilarity.

**Type:** China, Yunnan Province, Kunming City, Wild Duck Forest Park (25°13′ N, 102°87′ E, 2100 m above sea level), from the soil on the forest floor, 10 August 2019, Yao Wang (holotype: YHH 875, dried specimen; ex-type living culture: YFCC 875).

**Teleomorph:** Unknown.

**Anamorph:** Colonies on PDA are moderately fast-growing, attaining a diameter of 42–44 mm in 21 days at 22 °C. Colonies pulvinate, with high mycelial density, white or pale yellow, reverse deep yellow. Hyphae branched, smooth-walled, septate, hyaline, 1.1–2.7 µm wide. Cultures readily produce phialides and conidia on PDA after two weeks at room temperature. Phialides arising from aerial hyphae, solitary, 12.3–48.5 × 1.0–2.0 µm, cylindrical, tapering gradually toward the apex, neck 1.4–4.6 × 0.8–1.8 µm. Conidia hyaline, one-celled, globose to broadly ellipsoidal 1.8–3.4 × 1.3–1.9 µm. Chlamydospores present.

**Habitat:** Soil.

**Known distribution:** China.

**Additional specimens examined:** China, Yunnan Province, Kunming City, Songming County, Dashao Village (25°23′ N, 102°33′ E, 2700 m above sea level), from the soil on the forest floor, 12 August 2018, Yao Wang (living culture: YFCC 876).

**Comments:** Five species are closely related to *T. pseudoalbum* sp. nov., i.e., *T. pustulatum*, *T. tropicale*, *T. endophyticum*, *T. amazonense*, and *T. yunnanense* sp. nov. This clade is characterized by cylindrical to lageniform phialides, globose to broadly ellipsoidal conidia, and primarily white colonies. The phialides of *T. pseudoalbum* sp. nov. (12.3–48.5 × 1.0–2.0 µm) are longer than those of *T. album* (3.5–10 × 1.0–1.5 µm).

**Tolypocladium subparadoxum** H. Yu, Y. Wang and Q.Y. Dong, sp. nov., Figure 4.

**MycoBank:** MB 845431.

**Etymology:** Referring to the phylogenetic placement is closely related to *T. paradoxum*.

**Holotype:** China, Yunnan Province, Pu’er City, Simao District (22°43′ N, 100°58′ E, 1360 m above sea level), from soil on the forest floor, 27 August 2021, Yao Wang (holotype: YHH 879, dried specimen; ex-type living culture: YFCC 879).

**Teleomorph:** Not observed.

**Anamorph:** Colonies on PDA are moderately fast-growing, attaining a diameter of 36–38 mm in 21 days at 22 °C. Colonies flocculent, fluffy, with low mycelial density, white or pale yellow, reverse deep yellow. Hyphae smooth-walled, branched, septate, hyaline, 0.8–2.2 µm wide. Cultures produce phialides and conidia on PDA after two weeks at room temperature. Phialides arising from aerial hyphae, solitary, or in verticils of two to four, 5.4–40.1 × 0.9–1.8 µm, cylindrical, tapering gradually toward the apex, neck 3.2–5 × 0.7–1.2 µm. Conidia hyaline, one-celled, ellipsoidal or globose, single or aggregating in heads at the apex of phialides, 2.6–6.5 × 1.0–2.9 µm. Chlamydospores not observed.

**Habitat:** Soil, larvae of cicada.

**Known distribution:** China, Japan.

**Additional specimens examined:** NBRC 106958, Niryo, Takatsuki-shi, Osaka Préfecture.

**Comments:** Our phylogenetic analysis indicates that *Tolypocladium subparadoxum* sp. nov. is closely related to *T. paradoxum*. The two strains (YFCC 879 and NBRC 106958) formed a distinct lineage. NBRC 106958 was firstly isolated from cicada in Japan by S. Ban (https://www.nite.go.jp/nbrc/catalogue/NBRCCatalogueDetailServlet?ID=NBRCandCAT=00106958, accessed on 28 August 2022) and subsequently isolated from soil in China (YFCC 879). Since no significant morphological differences were found between the Chinese collections and that of Japan (Supplementary Figure S1), we treated YFCC 879 and NBRC 106958 as *Tolypocladium subparadoxum*. *Tolypocladium paradoxum* was originally described as *Cordyceps paradoxa* by Kobayasi, which was a cicada pathogen that produces solitary, pale ochraceous to dark olivaceous, fleshy stromata with cylindrical asci, breaking into cylindrical part spores [76]. Morphologically, *T. subparadoxum* differs...
from *T. paradoxum* in the following aspects. Relatively, *T. paradoxum* has longer phialides measured 5.8–58.3 × 1.8–4.3 μm, broader neck (0.9–1.9 μm vs 0.7–1.2 μm), and minor conidia (2.3–4.8 × 1.9–5.2 μm vs 2.6–6.5 × 1.0–2.9 μm) (Supplementary Figure S1).

**Figure 4.** Morphology of *Tolypocladium subparadoxum* (YFCC 879, ex-type living culture). (A, B) Culture characteristics on PDA medium incubated at 22 °C for 21 days; (C–F) phialides and conidia. Scale bars: (A, B) = 10 mm; (C–E) = 50 μm; (F) = 20 μm.

*Tolypocladium subparadoxum* similar to *T. dujiaolongae* and sharing cicada host, solitary, or verticillate, cylindrical or conical phialides, globose to ovoid conidia, and conidia aggregating mostly in small heads, but the latter differs by its relatively shorter phialides (11–35 × 1.0–2.7 μm vs 5.4–40.1 × 0.9–1.8 μm) [19]. Our phylogenetic analysis inferred from ITS data (Figure 2) suggests that they represent two distinct species.

*Tolypocladium geodes* is also similar to *T. subparadoxum* in their soil habitats and ellipsoidal or globose conidia. However, *T. geodes* has relatively shorter phialides (5.6–12.4 × 1.4–2.4 μm) and somewhat minor conidia (1.9–2.4 × 1.6–2.0 μm) [5]. Molecular phylogenetic analyses (Figures 1 and 2) indicate that they are distinct species.

*Tolypocladium yunnanense* H. Yu, Y. Wang and Q.Y. Dong, sp. nov., Figure 5


\[ \text{Figure 5. Morphology of Tolypocladium yunnanense (YFCC 877, ex-type living culture). (A,B) Culture characteristics on PDA medium incubated at 22 °C for 14 days; (C–J) phialides and conidia; (K) chlamydospore. Scale bars: (A,B) = 10 mm; (C,H) = 10 μm; (D–G,J–K) = 20 μm.} \]

**MycoBank:** MB 845432.

**Etymology:** *Yunnanense* (Lat.) refers to the type locality (Yunnan, China).
Holotype: China, Yunnan Province, Kunming City, Wild Duck Forest Park (25°14’ N, 102°87’ E, 2080 m above sea level), from soil on the forest floor, 12 August 2018, Yao Wang (holotype: YHH 877, dried specimen; ex-type living culture: Y FCC 877).

Teleomorph: Unknown.

Anamorph: Colonies on PDA are moderately fast-growing, attaining a diameter of 44–46 mm in 21 days at 22 °C. Colonies pulvinate, with high mycelial density, whitish to orange-yellow, reverse deep yellow. Hyphae smooth-walled, branched, septate, hyaline, 1.0–2.4 µm wide. Cultures produce phialides and conidia on PDA after two weeks at room temperature. Phialides are usually curved, solitary, 7.6–62.6 × 0.9–2.3 µm, cylindrical, narrowing slightly or abruptly into a neck, 3–4.2 × 0.5–1 µm. Conidia hyaline, one-celled, elliptical to subglobose, 1.2–2.4 × 0.9–1.9 µm. Chlamydospores present.

Habitat: Soil.

Known distribution: China.

Additional specimens examined: China, Yunnan Province, Pu’er City, Simao District (22°42’ N, 100°57’ E, 1348 m above sea level), from soil on the forest floor, 7 October 2019, Yao Wang (living culture: Y FCC 878).

Comments: Tolypocladium yunnanense sp. nov. is characterized by its solitary cylindrical phialides (7.6–62.6 × 0.9–2.3 µm), elliptical to subglobose conidia (1.2–2.4 × 0.9–1.9 µm), and white colonies. The five-gene phylogenetic analysis suggested that T. yunnanense sp. nov. was closely related to five other species (T. pustulatum, T. tropicale, T. endophyticum, T. amazonense and T. pseudoalbium sp. nov.). Phylogenetic analyses of this clade using ITS sequences, for which more complete data were available, showed that T. yunnanense sp. nov. formed clade with T. album, T. pseudoalbium sp. nov., T. tropicale, T. amazonense, and T. endophyticum. Morphologically, Tolypocladium yunnanense sp. nov. has longer phialides than other species in this clade: Tolypocladium yunnanense sp. nov., 7.6–62.6 × 0.9–2.3 µm; T. pustulatum, 4–10 × 2–4 µm; T. tropicale, 4.6 × 1.5 µm; T. endophyticum, 4.1 × 1.6 µm; T. amazonense, 4.1 × 1.6 µm; T. pseudoalbium sp. nov., 12.3–48.5 × 1.0–2.0 µm, and T. album, 3.5–10 × 1.0–1.5 µm.

Key to Tolypocladium species worldwide
Key to Tolypocladium species worldwide

12a. Stromata was connected to the host through a rhizomorph-like structure .................................................. 13
12b. Stromata arising directly from the host, never rhizomorphic ........................................................................ 14
13a. Fertile part yellowish-green when young, turning olive-green as it matures, perithecia relatively smaller, 480–590 × 195–235 µm ................................................................. T. bacillisporum
13b. Fertile part reddish brown to olivaceous brown, perithecia larger, 600–800 × 250–500 µm ......................... T. ophioglossoides
14a. Fertile part black, yellow black, dark chestnut brown when dried ................................................................. 15
14b. Fertile part pale bluish to grayish blue ........................................................................................................ 16
15a. Perithecia ≤ 700 µm long .......................................................... T. salicatistipitatum
15b. Perithecia > 700 µm long (750–1000 × 250–300 µm) .................................. T. tenusiorum
16a. Perithecia relatively narrower, 567–697 × 206–248 µm, part spores smaller, 2–5 × 1.5–2 µm, stromata 1.5–3 cm long .................................................. T. flavonigrum
16b. Perithecia relatively wider, 500–700 × 250–350 µm, part spores larger, 10–18 × 2.5–4 µm, stromata 2.5–7 cm long .......................................................... T. japonicum
17a. Perithecia larger .......................................................................................................................... 18
17b. Perithecia smaller, 400 × 250 µm ........................................................................................................ 19
18a. Stromata 12 cm long, part spores very long, 40–65 µm long .......................................................... T. longisegmentatum
18b. Stromata shorter than 12 cm, part spores < 40 µm long ........................................................................ 19
19a. Part spores ≤ 8 µm long .......................................................... T. deliciatistipitatum
19b. Part spores > 8 µm long ................................................................................................................ 20
20a. Asci shorter than 300 µm (240–300 × 7–8 µm), perithecia relatively smaller (450–540 × 230–260 µm) ................................................................................................................ 20
20b. Asci longer than 300 µm, perithecia larger .......................................................................................... 21
21a. Stipe slender, 0.5–1.0 mm thick, yellowish green to olivaceous, stromata 1.5–2.5 cm long, part spores 2–5 × 1.5–2 µm .................................................. T. fractum
21b. Stipe 1–5 mm thick, dark brown, smooth or furfuraceous, stromata 5–7 cm long, part spores longer, 3–8 × 2 µm .................................................. T. valifforme
22a. Perithecia < 550 µm long (480–540 µm) .......................................................................................... T. deliciatistipitatum
22b. Perithecia ≥ 550 µm long .......................................................... T. virens
23a. Conidia < 2.5–4.5 µm diam ............................................................................................................ 23
23b. Conidia ≥ 2.5–4.5 µm diam (3.0–4.5 µm diam) ..................................................................................... 23
24a. Part spores < 3 µm wide ................................................................................................................ 24
24b. Part spores ≥ 3 µm wide (3.0–4.5 µm) .......................................................................................... 25
25a. Fertile part olive-brown to olive-black, perithecia relatively larger, 650–950 × 250–420 µm, asci wider, 350–540 × 10–12 µm, part spores cylindrical or somewhat fusoid, 8–25 × 2.5–3 µm .................................................................................................................. T. capitatum
25b. Fertile part purple-brown, blacker when older, perithecia smaller, 600–750 × 200–300 µm, asci slender, 350–500 × 8–10 µm, part spores filiform, spindle-shaped, 15–20 × 2–3 µm .................................................................................................................. T. rouxii
26a. Perithecia relatively shorter, 520–740 × 300–330 µm, part spores cylindrical, 3–7 × 2–3 µm, on cicada nymphs .................................................. T. dujiatolongae
26b. Perithecia relatively longer, 900–930 × 220–250 µm, part spores fusoid, 16–18 × 3 µm, on Elaphomyces .................. T. minazakiaense
27a. From multiple substrate/host ............................................................................................................. 28
27b. From only a type of substrate/host .................................................................................................... 29
28a. Phialides cylindrical ......................................................................................................................... 29
28b. Phialides ellipsoidal to subglobose ................................................................................................. 30
29a. Colonies white, conidia globose to ovalid (phialides 3.5–10 × 1–1.5 µm, conidia 3.5 × 1.5–2.0 µm) .................................................................................................................. T. album
29b. Colonies white to pale yellow, conidia ellipsoidal, globose or broadly ellipsoidal ........................... T. inusitaticapitatum
30a. Phialides 4–10 × 2–4 µm, conidia 2–3 × 1.5–2.5 µm .................................................................................. 30
30b. Phialides 5.4–40.1 × 0.9–1.8 µm, conidia larger, 2.6–6.5 × 1–2.9 µm .................................................. T. subparadoxum
31a. From substrate ................................................................................................................................. 31
31b. On insects .................................................................................................................................... 32
32a. Substrate is not fungus ..................................................................................................................... 33
32b. Substrate is fungus .......................................................................................................................... 45
33a. From plant tissue ............................................................................................................................. 34
33b. From soil ....................................................................................................................................... 37
(T. amazonense, T. endophyticum, T. ovalisporum, T. tropicale)
34a. Conidia relatively more minor (globose, 1.3 µm diam) ................................................................. T. endophyticum
34b. Conidia larger, diam > 1.3 µm ........................................................................................................ 35
35a. Conidia > 4 µm long (4.5–9.0 × 2.5–3.3 µm) ............................................................................... T. ovalisporum
35b. Conidia < 4 µm long ...................................................................................................................... 36
36a. Phialides 4.6 ± 1.2 × 1.5 ± 0.3 µm, conidia spherical, relatively smaller, 1.5–0.1 µm diam .......................... T. amazconense
36b. Phialides 4.6 ± 1.5 × 1.5 ± 0.3 µm, conidia spherical, relatively smaller, 1.5–2.0 µm diam .................................................. T. tropicale
37a. Phialides cylindrical .......................................................................................................................... 38
37b. Phialides subglobose or ellipsoidal .......................................................................................... 39
38a. Conidia ellipsoidal, globose or broadly ellipsoidal ........................................................................... 39
38b. Conidia asymmetrically flattened, with a minute apical .................................................. T. microsporum
39a. Colonies white ............................................................................................................................. 40
39b. Colonies white or pale yellow (Phialides 12.3–48.5 × 1.0–2.0 µm, conidia smaller, 1.8–3.4 × 1.3–1.9 µm) .................................................................................................................. T. pseudoalbum
40a. Phialides shorter, 5.6–12.4 × 1.4–2.4 µm, conidia 1.2–2.4 × 0.9–1.9 µm .................................................. T. geodes
40b. Phialides longer, 7.6–62.6 × 0.9–2.3 µm, conidia 1.2–2.4 × 0.9–1.9 µm .................................................. T. yunnanense
41a. Conidia only one type ...................................................................................................................... 42
41b. Conidia two types (macroconidia ellipsoidal or reniform, 2.3–4.2 × 1.3–2.3 µm, macroconidia: cylindrical, 10 × 2.4 µm) .................................................. T. tenuisporum
### Key to *Tolypocladium* species worldwide

| 42a. | Phialides relatively longer, 4.4–7.8 × 1.5–2.7 µm, conidia cylindrical, 2.6–4.1 × 0.8–1.3 µm, colonies white to pale cream | *T. nubicola* |
| 42b. | Phialides shorter, 2.8–3.5 × 2.0–3.0 µm, conidia broadly oval, 2.5–3 × 2.0–2.5 µm, colonies white | *T. terricola* |
| 43a. | On *Elaphomyces* | *T. guangdongense* |
| 43b. | From *Ophiocordyceps sinensis* | *T. extinguens* |
| 44a. | Conidia reniform, 1.0–3.2 × 0.7–1.6 µm | *T. reniformisporum* |
| 44b. | Conidia spherical, 1.4–3.6 µm diam, phialides 7.6–19.4 × 2.9–3.6 µm | *T. sinense* |
| 45a. | On mosquito larvae, conidia two types (ellipsoidal: 2.5–1.5–2 µm, subglobose to ellipsoidal, or kidney-shaped: 3.5–4 × 3–3.5 µm) | *T. tigrincola* |
| 45b. | On bdelloid rotifers, conidia only one type | *T. trigonosporum* |
| 46a. | Conidia like an equilateral triangle or less ellipsoidal, 2–3 × 1.3–1.7 µm, colonies white or pale yellow | *T. varium* |

### 4. Discussion

*Tolypocladium* is one of the most diverse fungal groups in terms of shape, substrate or host, and habitat range. Many new species have recently been added to *Tolypocladium* [11–14,73]. The present study described three new species (*T. pseudoalbum* sp. nov., *T. subparadoxum* sp. nov., and *T. yunnanense* sp. nov.) based on phylogenetic analyses and morphological characteristics. Phylogenetically, these three species fell within the *Tolypocladium* clade, while morphologically all three species possessed cylindrical phialides and ellipsoidal or globose conidia. It is challenging to distinguish species of *Tolypocladium* based only on morphological characteristics, because several species in this genus are morphologically cryptic [7,8,11]. Sexual morphological features are diverse: the ovoid perithecia may be superficial or completely immersed and part spores size varies [7,10]. However, the asexual morphological features are relatively simple.

Species of *Tolypocladium* play a significant role in a variety of artificial and wild ecosystems and may participate in antifungal, host–fungi, and insecticidal interactions [10,77]. Many species have been described in *Tolypocladium* based on host associations or morphology [11,12]. Over the past several decades, the increasing number of new fungal species being discovered globally has dramatically changed the classification of early-diverging fungi [78]. In most previous studies, the classification of *Tolypocladium* was developed based on morphological characteristics. However, the advent of molecular biology, which was an important scientific milestone, revolutionized the taxonomic characterization of this genus. Over the last few decades, the number of accepted species in *Tolypocladium* has doubled.

All 48 of the currently accepted species of *Tolypocladium* were included in the key developed in this study. However, because the sequence loci for many of these taxa were incomplete, only 27 species were included in the multigene phylogenetic analyses (Figure 1). The multilocus phylogenetic approach used in this study of the genus *Tolypocladium* shed considerable light on this influential group of fungi.

The ITS region is the most commonly used molecular marker for species delimitation in fungi. Schoch et al. proposed ITS as the standard barcode for fungi. That proposal will satisfy most fungal biologists, but not all [57,79,80]. Species-level identification of fungi has long been considered challenging. Carlson et al. reported that ITS has a low molecular variation in *Trametes* leading to poorly resolved phylogenies and unclear species boundaries, especially in the *T. versicolor* species complex [80]. The results of this study indicated that the ITS sequences did not help substantially to separate *Tolypocladium* species. However, the ITS sequences did help to resolve the phylogenetic relationships between *Tolypocladium* and related genera. The analyses of molecular phylogeny based on ITS sequences used in the current classification of the genus fungus are congruent with the higher genus clades inferred from these analyses. However, ITS sequence data are not likely to resolve species-level relationships or to delimitate closely related species and species complexes. Using the ITS phylogeny, it was still not possible to identify some species of *Tolypocladium* with confidence in the new classification system; the ITS region alone could not accurately identify species in *Tolypocladium*. For example, in the ITS phylogeny, *T. varium*
CBS 429.94 was inseparable from *T. inflatum* OSC 71235 and *T. inflatum* NBRC 31669, while *T. tundrense* CBS 569.84 was inseparable from *T. cylindrosporum* ARSEF 2920 and *T. cylindrosporum* YFCC 1805001 (Figure 2). In contrast, relationships among *Tolypocladium* species were highly resolved in the phylogeny based on the protein-coding gene *rpb1*. Multilocus sequence analyses provide additional information to better characterize species boundaries [81]. Therefore, we used both morphological and multilocus phylogenetic evidence to support the novelty of the new species described in this study and to ensure accurate species identifications.

*Tolypocladium extinguens* was first reported from New Zealand by Samson et al. The original description was based on only a single isolate [82]. *Tolypocladium extinguens* is characterized by its prolonged growth in pure culture and its subglobose to ellipsoidal, sometimes kidney-shaped, conidia [82]. Our phylogenetic analysis did not support the placement of this species in *Tolypocladium* due to long branch attraction in the phylogenetic tree. More taxa must be added to this analysis in future to clarify the phylogenetic position of this species.

*Tolypocladium* species are well-known medicinal fungi that are also plant endophytes, soil inhabitants, and insect pathogens [10,12]. Because many of species of fungi are present in the soil environment at some stage of their life cycle, this substrate is preferred by researchers for the isolation of *Tolypocladium*. At least eight species have been reported from the soil: *T. geodes, T. microsporum, T. nubicola, T. pseudoalbum* sp. nov., *T. subparadoxum* sp. nov., *T. terricola, T. tundrense*, and *T. yunnanense* sp. nov. In Asia (China, Japan, and Thailand), *Tolypocladium* species are mainly known from insects [19], and few studies have focused on *Tolypocladium* species in the soil and in plant roots. Recently, *Tolypocladium* species in Chinese soils were surveyed, but no new species were identified.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jof8111158/s1. Table S1: Pairwise genetic distance matrix of *Tolypocladium* species for *tef-1a* sequences. Table S2: Pairwise genetic distance matrix of *Tolypocladium* species for partial *ITS* sequences. Table S3: Pairwise genetic distance matrix of *Tolypocladium* species for partial *rpb1* sequences. Table S4: Pairwise genetic distance matrix of *Tolypocladium* species for partial *rpb2* sequences. Figure S1. Morphology of *Tolypocladium subparadoxum* NBRC 106958 and *Tolypocladium paradoxum* NBRC 100945.

**Author Contributions:** Conceptualization, Y.W.; methodology, Y.W.; software, Q.-Y.D.; validation, Z.-Q.W. and Y.-F.L.; formal analysis, Q.-Y.D.; investigation, Y.W.; resources, H.Y.; data curation, Z.-Q.W.; writing—original draft preparation, Y.W. and H.Y.; visualization, Y.W.; funding acquisition, Y.W. and H.Y. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the National Natural Science Foundation of China (31870017 and 32160005).

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

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets presented in this study can be found in GenBank. The accession numbers can be found in the article (Table 1).

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

**References**

1. Gams, W. *Tolypocladium*, eine Hyphomycetengattung mit geschwollenen Phialiden. *Persoonia* 1971, 6, 185–191.
2. Barron, G.L. Fungal parasites of rotifers a new *Tolypocladium* with underwater conidiation. *Can. J. Bot.* 1980, 58, 439–442. [CrossRef]
3. Barron, G.L. Two new fungal parasites of bdelloid rotifers. *Can. J. Bot.* 1981, 59, 1449–1455. [CrossRef]
4. Barron, G.L. Structure and biology of a new *Tolypocladium* attacking bdelloid rotifers. *Can. J. Bot.* 1983, 61, 2566–2569. [CrossRef]
5. Bisset, J. Notes on *Tolypocladium* and related genera. *Can. J. Bot.* 1983, 61, 1311–1329. [CrossRef]
62. Crous, P.W.; Wingfield, M.J.; Burgess, T.I.; Hardy, G.E.; Crane, C.; Barrett, S.; Cano-Lira, J.F.; Le Roux, J.J.; Thangavel, R.; Guarro, J.; et al. Fungal Planet description sheets: 469–557. Persoonia 2016, 37, 218–403. [CrossRef]

63. Johnson, D.; Sung, G.; Hywel-Jones, N.L.; Luangsa-Ard, J.J.; Bischoff, J.F.; Kepler, R.M.; Spatafora, J.W. Systematics and evolution of the genus Torrubia (Hypocreales, Ascomycota). Mycol. Res. 2009, 113, 279–289. [CrossRef]

64. Zhang, S.; Zhang, Y.J.; Liu, X.Z.; Zhang, H.; Liu, D.S. On the reliability of DNA sequences of Ophiocordyceps sinensis in public databases. J. Ind. Microbiol. Biotechnol. 2013, 40, 365–378. [CrossRef]

65. Wang, Y.B.; Nguyen, T.T.; Dai, Y.D.; Yu, H.; Zeng, W.B.; Wu, C.K. Molecular phylogeny and morphology of Ophiocordyceps undituberculata sp. nov. (Ophiocordycipitaceae), a pathogen of caterpillars (Noctuidae, Lepidoptera) from Yunnan, China. Mycol. Prog. 2018, 17, 745–753. [CrossRef]

66. Wen, T.C.; Zhu, R.C.; Kang, J.C.; Huang, M.H.; Tan, D.B.; Ariyawansha, H.; Hyde, K.D.; Liu, H.A.O. Polyphasic analysis of Ophiocordyceps sp. nov. from larvae of Phassus nodus (Hepialidae) in Huan Province, southern China. Phytopathol. 2013, 123, 41–50. [CrossRef]

67. Bischoff, J.F.; Sullivan, R.F.; Struve, L.; Hywel-Jones, N.L.; White, J.F. Resurrection of Blistung laticium and its exclusion from Polypeolina (Hyphomycetes, Deuteromycota) based on 28S rDNA sequence data. Mycotaxon 2003, 86, 433–444.

68. Wang, Y.B. Studies on Phylogeny of Polycephalomyctaceae Fam. nov., with Microbial Diversities of Polycephalomyces multiramosus and Its Host. Ph.D. Thesis, Yunnan University, Kunming, China, 2015.

69. Wang, W.J.; Wang, X.L.; Li, Y.; Xiao, S.R.; Kepler, R.M.; Yao, Y.J. Molecular and morphological studies of Paecilomyces sinensis reveal a new clade in clavicipitaceae fungi and its new systematic position. Syst. Biodivers. 2012, 10, 221–232. [CrossRef]

70. Perdomo, H.; Cano, J.; Gene, J.; Garcia, D.; Hernandez, M.; Guarro, J. Polyphasic analysis of Purpureocillium lilacinum isolates from different origins and proposal of the new species Purpureocillium lavendulum. Mycologia 2013, 105, 151–161. [CrossRef]

71. Luangsa-ard, J.J.; Hywel-Jones, N.L.; Samson, R.A. The polyphyletic nature of Paecilomyces sensu lato based on 18S-generated rDNA phylogeny. Mycologia 2004, 96, 773–780. [CrossRef]

72. Gazis, R.; Skaltsas, D.; Chaverri, P. Novel endophytic lineages of Tolypocladium provide new insights into the ecology and evolution of Cordyceps-like fungi. Mycologia 2014, 106, 1090–1085. [CrossRef]

73. Wijayawardene, N.N.; Dissanayake, L.S.; Li, Q.R.; Dai, D.Q.; Xiao, Y.P.; Wen, T.C.; Karunarathna, S.C.; Wu, X.X.; Zhang, H.; Tiberromm, S.; et al. Yunnan–Guizhou Plateau: A mycological hotspot. Phytotaxa 2021, 2019, 279–289. [CrossRef]

74. Kobayasi, Y.; Shimizu, D. Monographic studies of cordyceps 2, group parasitic on cicadae. Bull. Natl. Sci. Mus. 1963, 6, 286–314.

75. Wang, J.C.; Zhang, Z.Z.; Li, Z.L.; Wang, Y. Research Progress of Ophiocordyceps sinensis sp. nov. in public databases. J. Invertebr. Pathol. 2013, 37, 1963–1969. [CrossRef]

76. Voigt, K.; James, T.Y.; Kirk, P.M.; Santiago, A.; Waldman, B.; Griffith, G.W.; Fu, M.; Radek, R.; Strassert, J.F.H.; Wurzbacher, C.; et al. Early-diverging fungal phyla: Taxonomy, species concept, ecology, distribution, anthropogenic impact, and novel phylogenetic proposals. Fungal Divers. 2021, 109, 59–98. [CrossRef] [PubMed]

77. Raja, H.A.; Miller, A.N.; Pearce, C.J.; Oberlies, N.H. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. J. Nat. Prod. 2017, 80, 756–770. [CrossRef] [PubMed]

78. Carlson, A.; Justo, A.; Hibbett, D.S. Species delimitation in Trametes: A comparison of ITS, RPB1, RPB2 and TEF1 gene phylogenies. Mycologia 2014, 106, 735–745. [CrossRef] [PubMed]

79. Santos, L.; Alves, A.; Alves, R. Evaluating multi-locus phylogenies for species boundaries determination in the genus Diaporthe. PeerJ 2015, 5, e3120. [CrossRef]

80. Samson, R.A.; Soares, G.G. Entomopathogenic Species of the Hyphomycete Genus Tolypocladium. J. Invertebr. Pathol. 1984, 43, 133–139. [CrossRef]