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Synhelminthosporium gen. et sp. nov. and Two New Species of Helminthosporium (Massarinaceae, Pleosporales) from Sichuan Province, China

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Abstract: Helminthosporium is a polyphyletic genus in Massarinaceae (Pleosporales). Species of Helminthosporium are characterized by having septeate and erect conidiophores, acro-pleurogenous and distoseptate conidia with a ring-shaped scar at the base. During a survey of fungal diversity in Sichuan Province, China, six Helminthosporium-like isolates were collected from dead branches of unknown trees. Five barcodes, including ITS (ITS1-5.8S-ITS2), SSU, LSU, TEF1, and RP2B were amplified and sequenced. Morphological examination and multi-locus phylogenetic analyses revealed two new Helminthosporium species (H. chengduense sp. nov., and H. chinense sp. nov.), a new genus (Synhelminthosporium gen. nov.) with a type species Synhelminthosporium synnematoferum sp. nov., and two known species (Helminthosporium submersum and H. velutinum) within Massarinaceae. The new genus Synhelminthosporium differs from the phylogenetically closest genus Helminthosporium by producing synnematous conidiophores. This work expands our understanding of the diversity of Helminthosporium-like taxa in Sichuan Province, China.

Keywords: Ascomycota; Dothideomycetes; fungal taxonomy; morphology; multi-locus; phylogeny

1. Introduction

Fungi consist of a highly diverse lineage of eukaryotes with a huge estimated number of between 2.2 and 3.8 million species [1]. Investigating fungal diversity is vital in Assembling the Fungal Tree Of Life (AFToL) [2], which significantly enhances our understanding of the history of life and also strengthens our ability to explore and use fungal resources [3].

Helminthosporium is an old, species-rich genus erected by Link in 1809 [4]. In addition to Helminthosporium, ten other genera are accepted in the family Massarinaceae (Pleosporales, Dothideomycetes), i.e., Byssothecium, Haplohelminthosporium, Helminthosporiella, Massarina, Mirohelminthosporium, Pseudodidymosphaeria, Pseudosplanchnonema, Seminispora, Stagonospora, and Suttonomyces [5,6]. Based on multi-locus phylogenetic analysis, Konta et al. [7] confirmed that Helminthosporium is polyphyletic, where members were mixed with other taxa of Byssothecium, Helminthosporiella, and Pseudosplanchnonema. Most Helminthosporium species are saprobes feeding on dead or decaying woods [7,8]. However, one species, H. solani is an economically important pathogen causing silver scurf disease in potatoes worldwide [9,10]. Helminthosporium species are commonly collected from leaves and decaying wood in terrestrial habitats [7,8,11] and rarely reported in freshwater habitats [12].
The genus *Helminthosporium*, typified by *H. velutinum*, is characterized by producing macroconidial, cylindrical, rather straight, septate, erect conidiophores with tretic conidiogenous cells and clavate or obclavate, distoseptate conidia with a flat, ringed pore at the base [8,13]. Conidia are produced mainly laterally from tretic conidiogenous cells and the production of a terminal conidium usually determines the end of conidiophore growth. Most *Helminthosporium* species were introduced based on their asexual morph and only six species, viz., *H. tiliae*, *H. microsorum*, *H. oligosporum*, *H. massarimum*, *H. quercicolae*, and *H. quercinum*, were characterized based on both morphs [8,11]. *Splanchnonema kalakadense* was described as the sexual morph of *H. velutinum*, but this was only based on pure culture without sequence data [14]. Tanaka et al. [11] first connected the *Massarina*-like sexual morph and asexual morph of *H. massarimum*, which was confirmed based on pure culture and sequence data. Voglmayr and Jaklitsch [8] experimentally confirmed three *Splanchnonema*-like sexual morphs of *Helminthosporium* species based on pure culture, sequence data, and herbarium studies, which extends the knowledge of sexual morphs of *Helminthosporium*.

Sichuan Province, located in southwestern China, along the Yangtze River, has enormous fungal diversity [15–18]. We regularly conduct fungal diversity surveys in Sichuan Province. During the study of preliminary morphological examination and BLASTn analysis of ITS sequences (the ribosomal internal transcribed spacer), a total of six *Helminthosporium*-like isolates were obtained from July to September 2021. Based on the multilocus phylogenetic analysis and morphological examination, two known *Helminthosporium* species including a new habitat record, two new *Helminthosporium* species, and a new genus *Synhelminthosporium* with the type species, *S. synnematoferum* sp. nov. are introduced. This study broadens our understanding of the diversity of *Helminthosporium*-like taxa.

2. Materials and Methods

2.1. Sample Collection, Isolation, and Morphological Examination

A survey of the diversity of ascomycetous fungi in Sichuan Province, China, was conducted between July and September 2021. Dead branches were collected from three locations in Sichuan Province (Yunqiao Wetland, Chengdu City; Baiyungou, Chongzhou City; Huilonggou, Pengzhou City). The specimens were taken to the laboratory in paper envelopes for examination. The morphological observation was consistently carried out from material on natural substrates. Tiny pieces of mycelium were mounted in a drop of sterilized water using syringe needles. Microscopic characters were observed and recorded using a Nikon SMZ800N stereo microscope equipped with a Nikon DS-Fi3 microscope camera and a Nikon ECLIPSE Ni-U microscope fitted with a Nikon DS-Ri2 microscope camera, respectively. Measurements were conducted using the Nikon NIS-Elements Documentation Imaging Software Version 5.21.00. All photos were processed using Adobe Photoshop software version 22.0. Isolates were obtained by picking up pieces of mycelium into sterilized water, spreading the suspension onto the surface of potato dextrose agar (PDA) plates, and incubated for 24 h at 25 °C. Germinated conidia were individually transferred to PDA plates and incubated under the dark at 25 °C. Culture characteristics were examined and recorded after one week and later at regular intervals.

The specimens were deposited in the Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS), Kunming, China, or the Herbarium of University of Electronic Science and Technology (HUEST), Chengdu, China. The living cultures were deposited in the China General Microbiological Culture Collection Center (CGMCC) in Beijing, China, and the University of Electronic Science and Technology Culture Collection (UESTCC) in Chengdu, China.
2.2. DNA Extraction, PCR Amplification, and Sequencing

Fungal genomic DNA was extracted from mycelia using the Trelief™ Plant Genomic DNA extraction Kit (TSINGKE Biotech, Shanghai, China) according to the manufacturer protocol. The DNA was stored at −20 °C for long-term storage. Five barcodes including the nuclear ribosomal internal transcribed spacer region (ITS: ITS1-5.8S-ITS2), the partial nuclear ribosomal small subunit rRNA gene (SSU), the partial nuclear ribosomal large subunit rRNA gene (LSU), the partial translation elongation factor 1-alpha gene (TEF1) and the partial second-largest subunit of RNA polymerase II gene (RPB2) were amplified by polymerase chain reaction (PCR). The corresponding primer pairs and PCR processes are listed in Table 1. The final PCR reaction volume was 25 μL containing 2 μL of DNA template, 1 μL each of the forward and reverse primer (10 μM), 8.5 μL of double-distilled water (ddH2O), and 12.5 μL of 2 × Flash PCR MasterMix (mixture of DNA Polymerase, dNTPs, Mg2+ and optimized buffer; CoWin Biosciences, Taizhou, China). The PCR products were visualized in 1% agarose gel electrophoresis. Sanger sequencing was conducted by Tsingke Biological Technology (Beijing, China).

Table 1. Loci used in this study with the corresponding PCR primers and conditions.

| Locus | PCR Primers | PCR: Thermal Cycles | Reference |
|-------|-------------|---------------------|-----------|
| ITS   | ITS9mun/ITS4_KYO1 | (94 °C: 30 s, 56 °C: 30 s, 72 °C: 30 s) × 35 cycles | [19] |
| LSU   | LR0R/LR5    | (94 °C: 30 s, 56 °C: 30 s, 72 °C: 1min) × 35 cycles | [20,21] |
| SSU   | PNS1/NS41   | (94 °C: 30 s, 56 °C: 30 s, 72 °C: 1min) × 35 cycles | [22] |
| TEF1  | EF1-728F or EF1-983/EF1-2218R or TEF1LLErev | (94 °C: 30 s, 52 °C: 30 s, 72 °C: 1min) × 35 cycles | [23–25] |
| RPB2  | dRPB2-5f or RPB2-5f2/ dRPB2-7r or fRPB2-7cR | (94 °C: 30 s, 52 °C: 30 s, 72 °C: 1min) × 35 cycles | [26–28] |

2.3. Phylogenetic Analyses

According to the corresponding Sanger sequencing chromatograms, misleading data from the ends of raw sequencing fragments were manually trimmed and assembled into consensus sequences using SeqMan Pro version 7.1.0 (DNASTAR, Inc. Madison, WI, USA). Barcode sequences of all Helminthosporium species currently available in GenBank, representative strains from other genera in Massarinaeaceae, and the outgroup taxon Periconia pseudodigitata (CBS 139699) were downloaded from the NCBI nucleotide database using the function read.GenBank integrated within the R package Analysis of Phylogenetics and Evolution (APE) [29].

The multiple sequence alignment was conducted using MAFFT version 7.310 [30] with options "--adjustdirectionaccurately--auto", and the alignment results were further trimmed using trimAl version 1.4 [31] with the option "--gapthreshold 0.5", which only allows 50% of taxa with a gap in each site. The best-fit nucleotide substitution models for each alignment dataset were selected using PartitionFinder version 2.1.1 [32] under the Corrected Akaike Information Criterion (AICC).

Maximum Likelihood (ML) and Bayesian analysis were conducted based on individual and combined datasets. Five alignment datasets of SSU, ITS, LSU, TEF1, and RPB2 were concatenated using an in-house python script for multi-locus phylogenetic analysis. ML phylogenetic trees were obtained using the IQ-TREE version 2.0.3 [33], and the topology was evaluated using 1000 ultrafast bootstrap replicates. The Bayesian analysis was conducted using parallel MrBayes version 3.2.7a [34]. Two different runs with 20 million generations and four chains were executed, and the initial 25% of sample trees were treated as burn-in. Tracer version 1.7.1 [35] was used to confirm that the MCMC runs reached convergence with all ESS values above 200. Then, the ML tree was annotated by TreeAnnotator version 2.6.6 implemented in BEAST version 2.6.6 [36] based on MrBayes.
MCMC trees with no discard of burn-in, and no posterior probability limit. The ML trees were visualized using ggtree [37] and further edited in Adobe Illustrator version 16.0.0.

3. Results

3.1. Molecular Phylogeny

Five barcode sequences were obtained successfully except for RPB2 of *H. velutinum* (UESTCC 22.0022) and *H. chengduense* sp. nov. Newly generated sequences were deposited in GenBank and the accession numbers are listed in Table 2. The combined dataset (ITS:1-557, SSU:558-1594, LSU:1595-2485, TEF1:2459-3719, RPB2: 3720-4836) was composed of 1744 distinct patterns, 1138 parsimony-informative sites, 325 singleton sites and 3373 constant sites. Five single-locus datasets ITS, SSU, LSU, RPB2, and TEF1 contained 258, 69, 106, 353, and 357 parsimony informative sites, respectively. The best-fit evolution models were GTR+I+G for the ITS, LSU, TEF1 and RPB2 partitions and HKY+G for the SSU partition.

| Organism                          | Culture/Specimen No. 1 | SSU 2 | LSU 2 | ITS 2 | RPB2 2 | TEF1 2 |
|-----------------------------------|-------------------------|-------|-------|-------|--------|--------|
| *Byssothecium aquaticum*          | CBS 675.92              | GU205235 | GU205217 | OM337536 | DQ767464 | GU349061 |
| *Haplohelmithosporium calami*     | MFLUCC 18-0074 HT       | MT928160 | MT928156 | MT928158 | – 2      | – 2    |
| *Helminthosporiella stilbacea*   | COAD 2126               | –      | –      | MG668862 | –      | MG682500 |
| *H. stilbacea*                    | MFLUCC 15-0813 HT       | MT928161 | MT928157 | MT928159 | –      | MT928151 |
| *H. stilbacea*                    | CPHmZC-01               | –      | KX228355 | KX228298 | –      | –      |
| *Helminthosporiella aquaticum*    | MFLUCC 15-0357 = S-096 HT | KU697310 | KU697306 | KU697302 | –      | –      |
| *H. austriacum*                   | CBS 139924 = L132 HT    | KY984420 | KY984301 | KY984301 | KY984365 | KY984437 |
| *H. austriacum*                   | CBS 14238 = L169        | –      | KY984303 | KY984303 | KY984367 | KY984439 |
| *H. austriacum*                   | L137                    | –      | KY984302 | KY984302 | KY984366 | KY984438 |
| *H. caespitosum*                  | CBS 484.77 = L99 ET     | KY984421 | JQ044448 | JQ044429 | KY984370 | KY984440 |
| *H. caespitosum*                  | L141                    | –      | KY984305 | KY984305 | KY984368 | –      |
| *H. caespitosum*                  | L151                    | –      | KY984306 | KY984306 | KY984369 | –      |
| *H. chengduense*                  | UESTCC 22.0024 = YQ 071048 = CGMCC | ON557757 | ON557745 | ON557751 | ON563073 | ON600598 |
| *H. chengduense*                  | UESTCC 22.0025 = YQ 071047 | ON557756 | ON557744 | ON557750 | ON563072 | ON600597 |
| *H. chiangraense*                 | MFLUCC 21-0087 HT       | –      | MZ538538 | MZ538504 | –      | –      |
| *H. chlorophorae*                 | BRIP 14521              | –      | –      | AF120259 | –      | –      |
| *H. dalbergiae*                   | MAFF 243853 = H 4628 = TS 36 | AB797231 | AB807521 | LC014555 | –      | AB808497 |
| *H. endiandrae*                   | CBS 138902 = CPC 22194 HT | –      | KP004478 | KP004450 | –      | –      |
| *H. erythrinicola*                | CPC 35291 = CBS 145569 HT | –      | MK876432 | NR_165563 | MK876486 | –      |
| *H. genistae*                     | CBS 142597 = L142 ET    | –      | KY984310 | KY984310 | KY984374 | –      |
| *H. genistae*                     | CBS 139922 = L129       | KY984423 | KY984309 | KY984309 | KY984373 | –      |
| *H. genistae*                     | CBS 139921 = L128       | KY984422 | KY984308 | KY984308 | KY984372 | –      |
| *H. hispanicum*                   | CBS 136917 = L109 HT    | KY984424 | KY984318 | KY984318 | KY984381 | KY984441 |
| *H. juglandinum*                  | CBS 136922 = L118 HT    | –      | KY984321 | KY984321 | KY984384 | KY984444 |
| *H. juglandinum*                  | CBS 136911 = L97        | KY984425 | KY984322 | KY984322 | KY984385 | KY984445 |
| Species               | Accession Numbers                                                                 |
|----------------------|-----------------------------------------------------------------------------------|
| H. juglandinum       | CBS 136912 = L101  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. juglandinum       | CBS 136913 = L102  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. leucadendri       | CBS 135133 = CPC 19345 HT  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. magnisporum       | MAFF 239278 = \text{H} 4627 = \text{TS} 33 HT  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. massarina         | CBS 139690 = \text{JCM 13095} = \text{MAFF} 239605 = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. massarina         | JCM 13094 = \text{MAFF} 239604 = \text{KT} 838 EP  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. microsorum        | CBS 136910 = L96 ET  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. microsorum        | L94  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. microsorum        | CBS 136916 = L108  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. microsorum        | L95  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. nanjingensis      | HHAUF020380 = ZM020380  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. oligosporum       | CBS 136909 = L93 ET  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. oligosporum       | CBS 136908 = L92  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. quercinum         | CBS 112393  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. quercinum         | CBS 136915 = L107  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. solani            | CBS 365.75  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. solani            | CBS 640.85  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. submersum         | MFLUCC 16-1360 HT  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. submersum         | MFLUCC 16-1290 PT  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. submersum         | UESTCC 22.0021 = Sara 08_3 = CGMCC  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. velutinum         | UESTCC 22.0022 = BY 14_2 = CGMCC  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |
| H. velutinum         | UESTCC 22.0026 = YQ 071,005 = CGMCC  \[\text{JCM 13095} = \text{MAFF} 239605] = \text{CBS} 135133 = \text{CPC} 19345 HT \[\text{MFLUCC} 16-1360 HT] |

**Note:** The table above lists species and their corresponding accession numbers, along with some additional information such as the genus names, species names, and疣other identifiers. The numbers and symbols in the table represent unique identifiers for each accession. The table is formatted to clearly display the information in a readable manner, with each species' name followed by a list of its corresponding accession numbers. The table includes entries for a variety of species, each with a unique set of accession numbers, indicating the diversity and specificity of the data. The use of symbols and numbers in the table helps in accurately identifying and tracking each species. This structured format makes it easy to compare and analyze the information for each species represented in the document.
| Species                                      | Accession Numbers                                      | Bootstrap | Jackknife |
|----------------------------------------------|--------------------------------------------------------|-----------|-----------|
| *Periconia pseudodigitata*                   | KT 1395 = HHUF 29370 = CBS 139699 = MFLUCC 195746     | 96         | 97         |
| Pseudodidymosphaeria spartii                 | MFLUCC 13-0273                                         |           |           |
| P. spartii                                   | MFLUCC 14-1212                                         |           |           |
| Pseudosplanchnonema phorcioides              | L16 = CBS 122935                                       |           |           |
| S. pseudopaludosa                            | MFLUCC 13-0533 = CGMCC 3.17583                         |           |           |
| S. pseudopaludosa                            | MFLUCC 13-0611                                         |           |           |
| S. pseudopaludosa                            | MFLUCC 14-0618                                         |           |           |
| S. imperfecticola                            | MFLUCC 15-0026 = ICMP 21563 1 HT                      |           |           |
| S. multiseptata                              | MFLUCC 15-0449 = ICMP 21562 1 HT                      |           |           |
| S. paludosa                                  | CBS 135085 = S601 FT                                   |           |           |
| S. perfecta                                 | KT 1726A = JCM 13099 = MAFF 239609                  | 90         | 90         |
| S. perfecta                                 | CBS 135099 = S656 FT                                   |           |           |
| S. pseudocaricis                             | CBS 135132 = S610 FT                                   |           |           |
| S. pseudopululosa                            | CPC 22645 = CBS 136424 1 HT                           |           |           |
| S. pululosa                                  | CBS 135085 = S601 FT                                   |           |           |
| S. pululosa                                  | CBS 135099 = S656 FT                                   |           |           |
| Suttonomyces clematidis                      | MFLUCC 14-0240 = GUCC 18                              |           |           |
| S. rosei                                     | MFLUCC 15-0051 FT                                      |           |           |
| Synhelminthosporium symmatoferum             | UESTCC 22.0023 = HLG 072894 1 HT                       | 100       | 100       |
|                                             | CGMCC 3.23574                                         |           |           |

1 Isolates from type materials are marked with ET (epi-type), HT (holotype), NT (neotype), and PT (paratype). 2 Missing sequences are indicated by “−.” 3 Newly generated sequences are in bold.

The best-scoring ML consensus tree (lnL = −21,360.862) with ultrafast bootstrap values from ML analyses and posterior probabilities from MrBayes analysis at the node is shown in Figure 1. Helminthosporium species are mixed with species of other genera, viz. Byssothecium, Pseudosplanchnonema, and Haplohelminthosporium, suggesting the genus is polyphyletic. Six newly obtained Helminthosporium isolates represent five different clades. Helminthosporium submersum (UESTCC 22.0021) clustered with two *H. submersum* isolates (MFLUCC 16-1360 and MFLUCC 16-1290). Helminthosporium velutinum (UESTCC 22.0022) and the five other *H. velutinum* strains including the epi-type (CBS 139923) are grouped into a statistically well-supported clade (100/1.00). *Synhelminthosporium symmatoferum* (UESTCC 22.0023) is separate from *H. erythrinicola* (CBS 145569) with strong statistical...
support (100%/1.00). Two isolates (UESTCC 22.0025 and UESTCC 22.0024) of *H. chengduense* form a distinct clade sister to *H. hispanicum* with high support values (98/1.00). *Helminthosporium chinense* (UESTCC 22.0026) is a sister to *H. nanjingensis* (ZM 20380) with strong statistical support (100%/1.00).

**Figure 1.** Phylogram of the best ML tree based on a combined dataset (SSU, ITS, LSU, TEF1, and RPB2) of Massarinaceae. Novel isolates are indicated in dark blue. Isolates from type materials are
in bold. The ML ultrafast bootstrap values/Bayesian PP greater than 95%/0.95 are indicated at the respective nodes. The tree is rooted with *Periconia pseudodigitata* (CBS 139699) (Periconiaceae, Pleosporales).

### 3.2. Taxonomy

**Helminthosporium chengduense** Y.P. Chen & Maharach., sp. nov. (Figure 2).  
*MycoBank:* MB 844416  

**Etymology:** The name refers to Chengdu, the city where the fungus was collected.  
**Saprobic** on decaying wood in a damp environment. **Sexual morph:** Unknown. **Asexual morph:** Colony on natural substrate punctiform, black, hairy. *Mycelium* mostly immersed, towards the surface forming stroma-like aggregations of light to dark brown pseudoparenchymatous cells. *Conidiophores* 133–391 μm long (x = 252, n = 40), 8–15 μm wide (x = 12, n = 40) at the base, tapering to 7–11 μm (x = 9, n = 40) at the apex, arising solitarily or in small groups from the stroma cells, erect, simple, straight or flexuous, thick-walled, subcylindrical, smooth, pale to dark brown, paler near the apex, with well-defined small pores at the apex and rarely laterally beneath the upper 1–2 septa. *Conidiogenous cells* mono- to poly-tretic, cylindrical, integrated, terminal and intercalary, pale brown to brown, secession schizo-lytic. *Conidia* 41–251 × 8–13 μm (x = 120 × 10, n = 60), tapering to 2–6 μm (x = 4, n = 55) at the distal end, with a blackish brown 2–5 μm wide (x = 3, n = 25) scar at the base, obclavate, straight, flexuous, sigmoid, lunate or uncinate, thin-walled, smooth, grey-white to pale brown, 3–16-distoseptate (n = 52), with angular lumina; wall up to 2–4 μm thick (x = 3, n = 68).

**Material examined:** China, Sichuan Province, Chengdu City, Yunqiao Wetland, on decaying branch of unidentified host, N 30°52′32″, E 103°53′23″, elevation 570 m, 10 July 2021, Y.P. Chen, YQ 071048H (HKAS 124016, holotype), culture ex-type UESTCC 22.0024 = YQ 071.048 = CGMCC 3.23575; ibid., YQ 071047H (HUEST 22.0025, isotype), culture ex-isotype UESTCC 22.0025 = YQ 071047.

**Culture characteristics:** Colony on PDA 53 mm diam after 2 weeks in an incubator under dark conditions at 20 °C, pale green, irregular circular, surface velvety, with white and denser mycelium at the center, with creamy white, entire margin; reverse dark green at the center, pale green at the periphery, with growth rings.

**Notes:** The phylogenetic tree shows that the isolate UESTCC 22.0024 clusters with the ex-type strain of *H. hispanicum* (CBS 136917) [8]. However, the isolate UESTCC 22.0024 significantly differs from the holotype in the length of conidiophores (133–391 μm vs. 130–540 μm), the size of conidia (41–251 × 8–13 μm vs. 69–130 × 17–24 μm), the wall thickness of angular lumina (2–4 μm vs. 7 μm). In addition, *H. hispanicum* is fungicolous and grows on old conidiomata of *Juglanconis juglandina* [8], whereas *H. chengduense* (UESTCC 22.0024) is saprobic on decaying wood in damp environments. Konta et al. [7] summarized the morphological characteristics of 216 Helminthosporium species. Among them, only *H. asterinum, H. longisinuatum,* and *H. makilingense* produce larger conidia than *H. chengduense* UESTCC 22.0024. The conidia of *H. chengduense* (UESTCC 22.0024) are much shorter and narrower than *H. asterinum* (500–600 × 80 μm) [38]; shorter than *H. longisinuatum* (65–1000 μm) [39], but with longer conidiophores (133–391 μm vs. 20–75 μm) and a smaller number of distosepta (3–16 vs. 9–22); shorter than *H. makilingense* (100–300 μm) [40]. Considering the significant differences in morphology and molecular data, we introduce the isolate UESTCC 22.0024 as a new species *H. chengduense.*
Figure 2. Helminthosporium chengduense (HKAS 124016, holotype). (a,b) Colonies on the natural substrate; (c) Conidiophores with apical and lateral conidia; (d) Conidiophores and stroma cells; (e) Conidiophore bases and stroma cells; (f) Conidiophore; (g-i) Conidiophore with young apical conidia; (j) Conidiophore with a young lateral conidium; (k) Conidiophore with an apical conidium; (l,m) Culture on PDA after 2 weeks (back and forth); (n1–n22) Conidia. Scale bars: d, f = 100 μm; e,
Helminthosporium chinense Y.P. Chen & Maharachch., sp. nov. (Figure 3).
MycoBank: MB 844417

Etymology: The name refers to China, the country where the fungus was collected.

Saprobic on decaying wood in damp environment. Sexual morph: Colony on natural substrate effuse, black, hairy. Mycelium mostly immersed, towards the surface forming stroma-like aggregations of light to brown pseudoparenchymatous cells. Conidiophores 214–461 μm long (x = 326, n = 40), 8–16 μm wide (x = 11, n = 38) at the base, tapering to 6–10 μm (x = 8, n = 38) at the apex, arising solitarily or in fascicles from the stroma cells, erect, simple, straight or flexuous, thick-walled, subcylindrical, smooth, pale to dark brown, with well-defined small pores at the apex and rarely laterally beneath the upper 1–5 septa. Conidiogenous cells mono- to poly-tretic, cylindrical, integrated, terminal and intercalary, brown, secession schizo-lytic. Conidia 42–109 × 5–11 μm (x = 61 × 8, n = 35), tapering to 2–6 μm (x = 4, n = 35) at the distal end, with a blackish-brown 3–5 μm wide (x = 4, n = 27) scar at the base, obclavate, straight or flexuous, thin-walled, smooth, pale gray to brown, 4–10-distoseptate, with angular lumina; wall up to 1–3 μm thick (x = 2, n = 36).

Material examined: China, Sichuan Province, Chengdu City, Yunqiao Wetland, on decaying branch of palm trees, N 30° 52′ 32″, E 103° 53′ 23″, elevation 570 m, 10 July 2021, Y.P. Chen, YQ 071005H (HKAS 124017, holotype), culture ex-type UESTCC 22.0026 = YQ 071,005 = CGMCC 3.23570.

Culture characteristics: Colony on PDA 31 mm diam after 2 weeks in an incubator under dark condition at 20 °C, white, irregular circular, surface velvety, with a clear margin; reverse white, with clear margin.

Notes: The phylogenetic tree shows that the isolate UESTCC 22.0026 clusters with the ex-type strain (ZM 20380) of H. nanjingensis, which was introduced by Wang et al. [41] from dead branches of an unidentified tree in Nanjing City, Jiangsu Province, China. Our collection (HKAS 124017) shares similar morphological characteristics in the shape and color of conidiophores and conidia with the holotype (HSAUP 0198) [41] of H. nanjingensis on natural substrate. However, it differs from H. nanjingensis by having significantly shorter conidia (42–109 μm vs. 64.5–170.5 μm) and smaller number of disto-septa (4–10 vs. 6–17) [41]. The BLASTn analysis of ITS of our isolate UESTCC 22.0026 showed 98% identity (446/453 bp, no gap) with ex-type strain (ZM 20380) of H. nanjingensis. Helminthosporium nanjingensis produced yellow-green pigment on PDA media [41], but the isolate UESTCC 22.0026 does not produce pigment on PDA. Only the ITS sequence is available for H. nanjingensis. Therefore, we cannot compare the sequence difference of other barcodes. Thus, considering the difference in morphology and the ability to produce pigments, we describe the isolate UESTCC 22.0026 as a new species H. chinense.
Figure 3. Helminthosporium chinense (HKAS 124017, holotype). (a) Natural substrate; (b,c) Colonies on the substrate; (d) Colony and conidiophores; (e) Conidiophore with an apical conidium; (f) Conidiophore base and stroma cells; (g) Conidiophore with an apical pore; (h) Stroma cells; (i) Conidiophore; (j) Young conidium; (k-q) Conidia; (r) Old conidium. (s,t) Front view and back view of...
culture on PDA after 2 weeks. Scale bars: d = 100 μm, e, g, h, i = 20 μm, f, j = 10 μm. Scale bar j applies to k–r.

_Helminthosporium submersum_ Z.L. Luo & H.Y. Su, Phytotaxa 348(4): 274 (2018) (Figure 4).

_Saprobi c_ on decaying wood in damp environment. **Sexual morph:** unknown. **Asexual morph:** Colony on natural substrate superficial, effuse, hairy, dark brown to black, glistening. _Mycelium_ mostly immersed, composed of septate, unbranched, smooth, thick-walled hyphae, on the bark stroma-like aggregations of light to black dark brown pseudoparenchymatous cells at the hyphae base. _Conidiophores_ mono-nematous, erect, simple, straight or flexuous, unbranched, smooth, thick-walled, subcylindrical, pale to dark brown, paler near the apex, 130–570 μm long, 15–31 μm wide (μ = 21, n = 20) at the base, tapering to 9–12 μm (μ = 10, n = 20) at the apex, with well-defined small pores at the apex and laterally beneath the upper 1–5 septa. _Conidiogenous cells_ mono- to poly-tretic, integrated, terminal and intercalary, cylindrical, pale brown, secession schizo-lytic. _Conidium_ acropleurogenous, simple, obclavate, straight or flexuous, thin-walled, smooth, grey-brown to brown, 49–86 × 14–25 μm (μ = 70 × 18, n = 35), tapering to 6–12 μm (μ = 8, n = 35) at the distal end, with a blackish-brown 3–6 μm wide (μ = 5, n = 40) scar at the base, 1–12-distoseptate (n = 30), with angular lumina; wall up to 2–4 μm thick (μ = 3, n = 35).

**Material examined:** China, Sichuan Province, Dujiangyan City, Longchi National Forest Park, on decaying branch of an unidentified host, N 31°6’37”, E 103°33’55”, elevation 1168 m, 19 September 2021, W. Tian, Sarah 08_3H (HUEST 22.0021), living culture UESTCC 22.0021 = Sarah 08_3 = CGMCC 3.23571.

**Culture characteristics:** Colony on PDA 19 mm diam after 2 weeks in an incubator under dark conditions at 20 °C, white, irregular circular, surface velvety, with denser mycelium at the center and becoming sparser towards the edge, with unclear margin; reverse pale green at the center, with unclear white margin.

**Notes:** The phylogenetic tree showed that our isolate (UESTCC 22.0021) from decaying wood in a damp environment clustered with the ex-type strain (MFLUCC 16–1360) of _H. submersum_, which was introduced by Zhao et al. [42] from submerged wood in freshwater. Morphologically, our collection fits well with _H. submersum_ by having effuse, velvety, dark brown or black colonies, mono-nematous, straight or flexuous, unbranched, pale to dark brown conidiophores, and similar size (49–86 × 14–25 μm vs. 41–55 × 14.5–18.5 μm) conidia [42]. Based on the overlapping morphological characteristics and the multi-locus phylogenetic tree, we identify our isolate as _H. submersum_. This is the first report of _H. submersum_ isolated from decaying wood in terrestrial habitats.
Figure 4. Helminthosporium submersum (HUEST 22.0021). (a) Natural substrate; (b–d) Colonies on natural substrates; (e) Conidiophore; (f) Conidiophore apex; (g) Conidiophore, conidiophore apex, apical conidia and lateral conidia; (h,i) Conidiophore base and stroma cells; (j–x) Conidia; (y,z) Front view and back view of a colony on PDA after 2 weeks. Scale bars: e–g = 100 μm, h, i = 20 μm, j–l = 10 μm. Scale bar l applies to m–x.
Helminthosporium velutinum Link [as ‘Helmisporium’], Mag. Gesell. naturf. Freunde, Berlin 3(1–2): 10, Table 1.9 (1809) (Figure 5).

Saprobic on decaying wood in damp environment. Sexual morph: Unknown. Asexual morph: Colony on natural substrate effuse, black, hairy, glistening. Mycelium mostly immersed, on the surface forming stroma-like aggregations of light to dark brown pseudo-parenchymatous cells. Conidiophores 343–941 μm long (x = 715, n = 20), 12–30 μm wide (x = 20, n = 20) at the base, tapering to 9–16 μm (x = 107, n = 20) at the apex, arising solitarily or in fascicles from the stroma cells, erect, simple, straight or flexuous, cylindrical, thick-walled, smooth, dark to blackish brown, paler near the apex, with well-defined small pores at the apex and rarely laterally beneath the upper 1–11 septa. Conidiogenous cells mono- to poly-tretic, cylindrical, integrated, terminal and intercalary, brown, secession schizo-lytic. Conidia 53–99 × 13–22 μm (x = 76 × 17, n = 36), tapering to 5–8 μm (x = 7, n = 36) at the distal end, with a blackish-brown 3–5 μm wide (x = 4, n = 33) scar at the base, obclavate, straight or flexuous, thin-walled, smooth, pale brown, 6–13-distoseptate, with angular lumina; wall up to 2–5 μm thick (x = 4, n = 43).

Material examined: China, Sichuan Province, Chongzhou City, Baiyungou, on decaying branch of an unidentified host, N 30°47′56″, E 103°24′15″, elevation 990 m, 27 September 2021, Y.P. Chen, BY 14_2H (HUEST 22.0022), living culture UESTCC 22.0022 = BY 14_2 = CGMCC 3.23572.

Culture characteristics: Colony on PDA 19 mm after 2 weeks in an incubator under dark conditions at 20 °C, creamy white, irregular circular, surface velvety, with denser mycelium at the center and becoming sparser towards the edge, with clear margin; reverse white, pale green in the center, with clear margin.

Notes: The phylogenetic tree showed that the isolate HUEST 22.0022 clustered with the isolates of H. velutinum. Helminthosporium velutinum, the type of the genus, is a well-known and most commonly recorded species [8]. It has been recorded mainly from woody substrates, and it is known for more than 100 host records [43]. Zhu et al. [12] first reported H. velutinum from a freshwater habitat in China, which is a less common habitat for this species. Our collection (HUEST 22.0022) displays similar morphological characteristics with the type of H. velutinum in the shape and color of colonies, conidiophores, conidiogenous cells, and conidia on the natural substrate [8]. We identified the isolate UESTCC 22.0022 as H. velutinum, a new record from terrestrial habitats in China considering similar morphological characteristics.
Figure 5. Helminthosporium velutinum (HUEST 22.0022). (a,b) Colonies on the natural substrate; (c) Conidiophores with apical and lateral conidia; (d,e,j) Conidiophores with stroma cells; (f) Conidiophore apex with an apical conidium; (g) Conidiophore with a lateral conidium; (h,i) Front view and back view of culture on PDA after 1 week; (k–z) Conidia. Scale bars: d–g, j, k, s = 20 μm. Scale bar k applies to l–r; s applies to t–z.
Synhelminthosporium Y.P. Chen & Maharachch., gen. nov.

Mycobank: MB 844418

Etymology: Syn = together with its close phylogenetic relationship with Helminthosporium.

Saprobic on decaying wood in damp environment. Sexual morph: Unknown. Asexual morph: Colony on natural substrate evenly distributed, hairy, glistening. Mycelium mostly immersed. Conidiophores synnematosous, compact, erect, straight or flexuous, unbranched, septate, smooth, subcylindrical, brown to dark brown, separated from the main body near the apex. Conidiogenous cells mono-tretic, cylindrical, integrated, terminal, brown, secession schizo-lytic. Conidia obclavate, straight or flexuous, smooth, pale brown, distoseptate, with angular lumina.

Type species: Synhelminthosporium synnematoferum Y.P. Chen & Maharachch. sp. nov.

Notes: The genus Synhelminthosporium is introduced based on the new species S. synnematoferum. Both BLASTn analysis results of five barcode sequences (SSU, ITS, LSU, TEF1, and RPB2) and multi-locus phylogenetic analyses showed that Synhelminthosporium is distinct and phylogenetically close to Helminthosporium. Synhelminthosporium differs from Helminthosporium by having synnematosous conidiophores, which is a character only presented in Helminthosporiella within Massarinaceae [7,44]. Phylogenetic analysis shows that Synhelminthosporium is different from Helminthosporiella. In addition, Helminthosporiella shows catenate conidia, but this character is absent in Synhelminthosporium. Based on distinguishing morphological characteristics and multi-locus phylogenetic analyses, we introduce a new genus Synhelminthosporium to accommodate the new species S. synnematoferum in Massarinaceae.

Synhelminthosporium synnematoferum Y.P. Chen & Maharachch., sp. nov. (Figure 6).

Mycobank: MB 844419

Etymology: The name refers to the synnematosus conidiophores.

Saprobic on decaying wood in damp environment. Sexual morph: Unknown. Asexual morph: Colony on natural substrate evenly distributed, hairy, glistening. Conidiophores 700–1500 μm long, synnematosous, compact, erect, straight or flexuous, unbranched, septate, smooth, subcylindrical, brown to dark brown, separated from the main body from the middle part up to near the apex. Conidiogenous cells mono-tretic, cylindrical, integrated, terminal, brown, secession schizo-lytic. Conidia 52–116 × 14–20 μm (x = 80 × 16, n = 36), tapering to 5–10 μm (x = 7, n = 36) at the distal end, with a blackish-brown 3–7 μm wide (x = 5, n = 36) scar at the base, obclavate, straight or flexuous, smooth, pale brown, 4–11-distoseptate (n = 35), with angular lumina; wall up to 2–4 μm thick (x = 3, n = 36).

Material examined: China, Sichuan Province, Pengzhou City, Huilonggou, on decaying branch of an unidentified host, N 31°11′6″, E 103°54′56″, elevation 1400 m, 28 July 2021, Y.P. Chen, HLG 072894H (HKAS 124015, holotype), culture ex-type UESTCC 22.0023 = HLG 072,894 = CGMCC 3.23574.

Culture characteristics: Colony 52 mm diam after 1 week in an incubator under dark condition at 20 °C, creamy white, irregular circular, surface velvety, with denser mycelium at the center and becoming sparser at the edge, with a clear margin; reverse creamy white, pale green at the center, with clear margin.

Notes: The best BLASTn matches of five barcode sequences (SSU, ITS, LSU, TEF1, and RPB2) of the isolate UESTCC 22.0023 belong to the genus Helminthosporium. The phylogenetic tree shows that our isolate (UESTCC 22.0023) clusters with the ex-type strain H. erythrinicola (CBS 145569). The BLASTn analysis of H. erythrinicola (CBS 145569) and S. synnematoferum (UESTCC 22.0023) shows 94% identity (538/570, 5 gaps) using ITS, 99% identity (820/831, 3 gaps) using LSU and 93% identity (822/884, no gap) using RPB2. Helminthosporium erythrinicola was introduced by Crous et al. [45], it is a typical Helminthosporium species with simple, multisepate, unbranched, brown conidiophores, tretic conidiogenous cells, and acro-pleurogenous, clavate or obclavate, distoseptate conidia. Our collection (HKAS 124015) differs from the H. erythrinicola and other Helminthosporium
species in having synnematous conidiophores. Synnematous conidiophores are only present in *Helminthosporiella* in Massarinaceae [7,44]. The isolate UESTCC 22.0023 is different from *Helminthosporiella* phylogenetically and morphologically. The absence of catenate conidia is the most prominent characteristic distinguishing *Synhelminthosporium synnematofерum* from *Helminthosporiella* species. The multi-locus phylogenetic tree shows the placement of *S. synnematoferus* (UESTCC 22.0023) within Massarinaceae, which is distinct from other known species.

Figure 6. *Synhelminthosporium synnematoferus* (HKAS 124015, holotype). (a–c) Colonies on natural substrates; (d) Synnematous conidiophores; (e) Conidiophores with apical conidia; (f) Conidioge-
nous cell with an apical pore; (g-i) Conidiogenous cells with apical conidia; (j,k) Conidiophore bases; (l) Young conidiom; (m-s) Conidia; (t,u) Front view and back view of culture on PDA after 1 week. Scale bars: d, j = 100 μm, e-i, k = 20 μm, l = 10 μm. Scale bar l applies to m–s.

4. Discussion

To date, there are 775 epithets of Helminthosporium (http://www.indexfungorum.org; April 2022), whereas many of them are not congeneric with the generic type, and were reclassified into other groups in subsequent studies [46–48]. For instance, H. cynodontis was reassigned to the genus Bipolaris (Pleosporaceae, Pleosporales) due to the production of sympodial conidiogenous cells and by having darkly pigmented conidiogenous loci [46]. Recently, Konta et al. [7] accepted 216 Helminthosporium species, however many species are identified only based on morphological studies, and only 25 species have sequence data. The lack of a large amount of molecular data is mainly because most species were introduced before the advent of Sanger sequencing. Considering that numerous Helminthosporium species were characterized only based on morphological studies, it is likely that some of them belong to the same species or even to different genera. During phylogenetic analysis, abnormal long branches were observed in four Helminthosporium strains with incorrect taxonomic positions, viz. H. asterinum (CBS 203.35), H. decacuminatum (CBS 185.47), H. anomalum (CBS 161.27), and H. gibberosporum (CBS 200.32). These species were introduced and characterized before the 1950s [49–52], whereas the sequence data related to them were submitted to GenBank by Vu et al. [53]. BLASTn analyses of these sequences showed that the top hits of ITS and LSU sequences for H. anomalum (CBS 161.27) belong to Bipolaris, ITS and LSU sequences for H. asterinum (CBS 203.35) belong to Kirschsteiniothelia, ITS and LSU sequences for H. decacuminatum (CBS 185.47) and H. gibberosporum (CBS 200.32) belong to Curvularia. The present study introduces two new Helminthosporium species and a new genus Synhelminthosporium based on multi-locus phylogenetic analysis and morphological studies. This phylogeny needs to be expanded by re-examining type materials of old described Helminthosporium-like species without molecular data, collecting new fresh specimens, sequencing, and using multi-locus analysis to establish epi-types or neotypes as necessary. Our new species can be distinguished from all other Helminthosporium species by morphological features and multi-locus phylogenetic analysis, and thus we are confident that the newly introduced species are distinct.

Recent studies have no universally accepted standard in selecting barcodes for phylogenetic analysis. Boonmee et al. [54] introduced H. chiangraicense using ITS and LSU. Crous et al. [45] introduced H. erythrinicola and H. szeygii using ITS, LSU, and RPB2. Zhu et al. [12] introduced H. aquaticum using SSU, ITS, and LSU barcodes. Voglmayr and Jaklitsch [8] pointed out that only ITS and/or LSU sequences can be problematic in resolving the phylogeny of Massarinaeae. Other barcodes RPB2 and TEF1 were proposed in multi-gene phylogenetic analyses of Massarinaeae, as these barcodes usually significantly increase the phylogenetic resolution [8,11]. However, the majority of Helminthosporium species do not have RPB2 and TEF1 barcodes (19 species have SSU sequence data; 25 species have LSU sequence data; 23 species have LSU sequence data; 17 species have RPB2 sequence data and 15 species have TEF1 sequence data). The present study conducted both individual and combined phylogenetic analyses. ITS, RPB2, and TEF1 barcodes offered more parsimony informative sites than SSU and LSU. In addition, more powerful resolution in delineating species and higher bootstrap support values for most clades were observed in single-gene ML trees (Figures S1 and S2), indicating that these barcodes are better in resolving genera in Massarinaeae than the other two barcodes.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/jof8070712/s1, Figure S1: Single-gene trees. Novel isolates are indicated in dark blue. Type isolates are in bold. The ML ultrafast bootstrap values/Bayesian PP greater than 95%/0.95 are indicated at the respective nodes. The tree is rooted with Periconia pseudodigitata (CBS 139699) (Periconiaceae, Pleosporales). Figure S2: Comparisons of the single-gene ML trees and the multi-locus ML tree in delineating species. (a) The proportion of highly supported internal
nodes, bootstrap values ≥ 95%. Protein-coding barcodes TEF1 and RP2B offer higher proportions of highly supported internal nodes than three nuclear ribosomal regions (b) Pairwise tree distances. The smaller the value, the higher the similarity in topology.

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