**Abstract:** A rare plant species of the *Orchidaceae* family, *Dendrobium officinale* is considered among the top ten Chinese medicinal herbs for its polysaccharide. Since 2021, when the dieback disease of *D. officinale* was first reported in Yueqing City, Zhejiang Province, China, *Fusarium* isolates (number = 152) were obtained from 70 plants in commercial greenhouses. The disease incidence ranged from 40% to 60% in the surveyed areas. Multilocus sequence analysis (MLSA) coupled with morphological characterization revealed that the collected isolates belonged to five species (sp.), viz., *Fusarium concentricum*, *F. fujikuroi*, *F. nirenbergiae*, *F. curvatum*, and *F. stilboides*, with isolation frequencies of 34.6%, 22.3%, 18.4%, 13.8%, and 10.5%, respectively. Notably, at least two *Fusarium* species were simultaneously isolated and identified from the infected plants. Finally, the pathogenicity test results demonstrated that such species were responsible for the dieback disease of *D. officinale*. However, *F. concentricum* and *F. fujikuroi* were more invasive compared to the other species in this study. To the best of the authors’ knowledge, this study was the first report of *F. concentricum, F. curvatum, F. fujikuroi, F. nirenbergiae*, and *F. stilboides* causing the dieback disease of *D. officinale* in China and worldwide. This work provides valuable data about the diversity and pathogenicity of *Fusarium* populations, which will help in formulating effective strategies and policies for better control of the dieback disease.

**Keywords:** dieback; MLSA; morphology; tiepishihu

---

1. **Introduction**

Commonly known as Tiepishihu, *Dendrobium officinale* (Kimura and Migo) is an epiphytic, herbaceous, flowering, insect-pollinated, and perennial plant with a cylindrical-fibrous stem that is mostly utilized for pharmaceutical purposes in traditional Chinese medicine (TCM) [1–3]. Thus far, it has been demonstrated that several bioactive constituents, comprising polysaccharides, alkaloids, phenanthrenes, bibenzyls, saccharides, glycosides, lignans, phenolic acids, and phenylpropanoids, possess a wide variety of pharmacological properties, including antioxidant, antitumor, and hypolipidemic activities, as well as anti-fatigue, hypoglycemic, anti-fibrotic, hepatoprotective effect, and immuno-enhancement enhancement effects, along with outcomes for rheumatoid arthritis and diabetes [1,3,4].

Tiepishihu is extensively cropped in several provinces of China including Zhejiang, Anhui, Fujian, Guizhou, Guangxi, Sichuan, and Yunnan, with a total cultivation area of nearly 4000 hectares [3–5], and it is valued between $450 and $3100 per kg. Recently, micropropagation and greenhouse farming technologies have been utilized to enhance the rate of low natural regeneration of this plant species [2,3,5]. The seedlings derived from tissue cultures are thus transplanted from March to May in greenhouses to produce tillers, and the flowers emerge about 15 months later, between May and July. The stems are further harvested from the fields from the end of October to the beginning of March, approximately 31 months after planting. For three years after the first harvest, the plant can also produce commercial yields (i.e., stems) annually.
Currently, *D. officinale* production is seriously threatened by a number of fungal genera such as *Alternaria alternata*, *Ceratobasidium* sp. (species), *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *C. fructicola*, *Epicoccum sorghinum*, *Fusarium equiseti*, *F. kyushuense*, *F. oxysporum*, *Neopestalotiopsis clavispora*, *Phoma multirostrata*, and *Sclerotium delphini* [6–12]. Among them, *F. equiseti* causes the dieback disease, which was initially identified in Fujian Province, China [8]. The pathogen results in considerable losses of approximately 50%. The infected plants exhibit wilting and dieback on apical meristem leaves, followed by drying and death of the tips [8].

Phytopathologists and clinical microbiologists have now devoted much attention to *Fusarium* (Wollenweber and Reinkin; Gibberella as sexual morphs) as a species-rich, heterogeneous, and ubiquitous genus of filamentous fungi [13–15]. In addition, the fusarioid taxa are able to adapt to a variety of climatic zones and colonize a wide range of ecosystems and hosts [13,16]. To date, this agriculturally and clinically important genus is estimated to be composed of over 400 phylogenetically distinct species, 23 monophyletic species complexes, and quite a few monotypic lineages [13,15,17]. The *F. buharicum* species complex (FBSC), *F. fujikuroi* species complex (FFSC), *F. incarnatum-equiseti* species complex (FIESC), *F. lateritium* species complex (FLSC), *F. oxysporum* species complex (FOSC), *F. redolens* species complex (FRSC), *F. sambucinum* species complex (FSaSC), and *F. solani* species complex (FSSC) have been accordingly reported to cause devastating plant diseases over the years [13,17–19]. Moreover, it has been difficult to discriminate closely related *Fusarium* species through macro-/microscopic characteristics due to the high morphological variability inter-/intra-species. Nowadays, several new species have been further delineated within the *Fusarium* spp. complexes by applying a polyphasic approach to taxonomy that combines morphology-based identification and genealogical concordance among portions of multiple phylogenetically informative genes (i.e., genealogical concordance phylogenetic species recognition: GCPSR sensu). This technique has thus led to a major improvement in the Fusarioid fungi taxonomy and nomenclature [13,16–20].

The dieback disease is spreading in different Tiepishihu plantation areas, but there are no effective and eco-friendly control measures available. In this context, integrated disease management strategies, including biological control agents, biofertilizers, chemical fungicides, cultural practices, resistant varieties, forecasting models, and sanitation methods are required to minimize the incidence rate of plant pathogens and maintain profitable, sustainable Tiepishihu production [21,22].

Hitherto, little has been known about the species, or about the population structure and phenotypic characters of *Fusarium* species causing the dieback disease of *D. officinale*, neither in China nor elsewhere in the world. Against this background, this study aimed to identify and characterize the *Fusarium* spp. diversity, and then to assess the virulence of the disease for Tiepishihu.

2. Materials and Methods

2.1. Sample Collection, Fungal Isolation, and Morphological Characterization

The *D. officinale* plants (70) with dieback symptoms were initially sampled from commercial greenhouses in Yueqing (28.07° N, 120.57° E), Zhejiang Province, China. The incidence rate of the disease was assessed by visual observations, and then calculated for the presence or absence of symptomatic plants in the surveyed greenhouses. Afterward, the symptomatic stem tips were cut with a sterilized scalpel, superficially disinfected with a 2% solution of sodium hypochlorite (0.1% active ingredient of chlorine; [23]) for 1 min and 75% ethanol for 30 s, rinsed thrice with sterile distilled water, air dried on sterile filter papers under aseptic conditions, and finally placed onto potato dextrose agar (PDA) medium. The plates were subsequently incubated at 25 °C in the dark, and the colonies were purified by the hyphal tip method and then sub-cultured on the PDA and carnation leaf agar (CLA) media for morphological observation [13,24]. The conidial morphology and sporulation of the pure fungal colonies were finally examined under a Nikon Eclipse microscope (Japan).
2.2. DNA Sequencing and Molecular Phylogeny

DNA was extracted from the mycelia of 7-day-old cultures of the representative isolates using the Plant Genomic DNA kit (Tiangen, China) according to the manufacturer’s instructions. The fragments of the translation elongation factor 1-alpha (tef1), second largest subunit of RNA polymerase II gene (rpb2), and β-tubulin (tub2) genes were then amplified by the primers EF-1/EF-2, RPB2-5f2/RPB2-7cr, and Tub2F/Tub2R, respectively \[13,23\]. The polymerase chain reaction (PCR) was also performed in 25 µL volumes, containing 1 µL of genomic DNA, 12.5 µL 2 × Phanta™ Flash Master Mix Dye Plus (Vazyme, Nanjing, China), 9.5 µL of DNase-free water, and 1 µL of each forward and reverse primer (10 µM). Notably, the cycling conditions included the initial denaturation of 30 s at 98 °C, followed by 30 cycles of denaturation at 98 °C for 10 s, the annealing at 52 (tef1), 59 (rpb2), and 55 (tub2) for 10 s, the extension of 10 s at 72 °C, as well as the final extension at 72 °C for 1 min. The PCR products were first visualized on a 1% (w/v) agarose gel, and then Sanger sequencing was conducted by Sangon Biotech Co., Ltd. (Shanghai, China) for both directions to ensure high accuracy. The accession number of all generated sequences in this study was further obtained from the GenBank, as listed in Table 1. The aligned sequences of the novel isolates were also subjected to the Basic Local Alignment Search Tool (BLAST) to collect related sequences for inclusion in phylogenetic analysis. The BLASTN searches were fulfilled using the rpb2, tef1, and tub2 sequences against the Nucleotide collection (nr/nt) database by restricting the material type. Multiple sequence alignments were correspondingly inferred in Molecular Evolutionary Genetics Analysis (MEGA) X software version 10.2.4 \[25\] using the MUSCLE (multiple sequence comparison by log-expectation) program \[26\] and refined manually if necessary. To generate concatenated datasets, single-gene sequences (tef1, rpb2, and tub2) were manually combined utilizing the BioEdit version 7.1 \[27\]. The phylogenetic trees were further constructed based on the individual and concatenated sequences (rpb2, tef1, and tub2) using the MEGA X software. The maximum likelihood (ML) and neighbor-joining (NJ) methods were also employed to approximate the distances and complete bootstrapping. As well as the general time reversible model assuming a discrete gamma distribution and invariant sites (GTR+I+G) for the combined aligned dataset, the Tamura-Nei model with gamma-distributed (TN93+G) for rpb2 and the Kimura two parameter model (K2+G) for tef1 were applied as the best evolutionary models for the phylogenetic analyses \[25\]. The topological support was then determined by 1000 bootstrap replicates. The sequences from the Fusarium spp. type strains, initially identified as closely related to the sequences here, were finally included by the preliminary BLAST searches (Table 1).

Table 1. GenBank accession numbers of Fusarium strains used in the phylogenetic analyses.

| Species          | Culture Collection No./Isolate | GenBank Accession |
|------------------|--------------------------------|-------------------|
|                  |                                | rpb2              |
| Fusarium carminascens | CPC 144738 T          | MH484937          |
| F. contaminatum  | CBS 111552 T          | MH484901          |
| F. pharetrum     | CBS 144751 T          | MH484952          |
| F. veterinarium  | CBS 109898 T          | MH484899          |
| F. cugenangense  | CBS 620.72            | MH484879          |
|                  | FC4                  | MH484983          |
| F. curvatum      | FC6                  | MH484984          | MH485075 |
| F. fabacearum    | CBS 25802 T          | MH484939          |
| F. glycines      | CBS 144746 T          | MH484942          |
| F. gossypinum    | CBS 116613 T          | MH484909          |
| F. languescens   | CBS 645.78 T          | MH484880          |
Table 1. Cont.

| Species            | Culture Collection No./Isolate | GenBank Accession rpb2 | GenBank Accession tef1 | GenBank Accession tub1 |
|--------------------|-------------------------------|------------------------|------------------------|------------------------|
| *F. libertatis*    | CPC 28465 T                   | MH484944               | MH485035               | MH485126               |
|                    | CBS 840.88 T                  | MH484887               | MH484978               | MH485069               |
| *F. nirenbergiae*  | FNS1                          | ON137574               | ON137553               | ON137595               |
|                    | FNS3                          | ON137575               | ON137553               | ON137596               |
|                    | FNS10                         | ON137576               | ON137553               | ON137597               |
| *F. oxysporum*     | CBS 144134 ET                 | MH484953               | MH485044               | MH485135               |
| *F. hoodiae*       | CBS 132474 T                  | MH484929               | MH485020               | MH485111               |
| *F. duoseptatum*   | CBS 102026 T                  | MH484896               | MH484987               | MH485078               |
| *F. callistephi*   | CBS 187.53 T                  | MH484875               | MH484966               | MH485057               |
| *F. triseptatum*   | CBS 258.50 T                  | MH484910               | MH485001               | MH485055               |
| *F. langsuescens*  | CBS 645.78 T                  | MH484880               | MH484971               | MH485062               |
| *F. elaeidis*      | CBS 217.49                    | MH484870               | MH484961               | MH485052               |
| *F. acutatum*      | CBS 402.97 T                  | MW402768               | MW402125               | MW402323               |
| *F. agapanthi*     | NRRL 54463 T                  | KU900625               | KU900630               | KU900635               |
| *F. ananatum*      | CBS 118516 T                  | LT996137               | LT996091               | MN534089               |
| *F. andipaci*      | CBS 119857 T                  | LT996138               | MN193854               | LT996113               |
| *F. annulatum*     | CBS 258.54 T                  | MT010983               | MT010994               | MT011041               |
| *F. anthophilum*   | CBS 222.76 ET                 | MW402811               | MW402114               | MW402312               |
| *F. bactridioides* | CBS 1405.97 T                 | MN534295               | MN533995               | MN534112               |
| *F. brevicatenulatum* | CBS 404.97 T   | MN534295               | MN533995               | MN534063               |
| *F. bulbicola*     | CBS 220.76 T                  | MW402767               | KF466415               | KF466437               |
| *F. chinhoiensis*  | NRRL 25221 T                  | MN534262               | MN534050               | MN534082               |
| *F. subglutinans*  | CBS 747.97 NT                 | MW402773               | MW402150               | MW402351               |
| *F. circinatum*    | CBS 405.97 T                  | MN534252               | MN533997               | MN534097               |
| *F. coicis*        | NRRL 66233 T                  | KP083274               | KP083251               | LT996115               |
|                    | CBS 450.97 T                  | JF741086               | AF160282               | MW402334               |
| *F. concentricum*  | FCZ2                          | ON137559               | ON107278               | ON137580               |
|                    | FCZ8                          | ON137560               | ON107279               | ON137580               |
|                    | FCZ25                         | ON137561               | ON107280               | ON137582               |
|                    | FCZ30                         | ON137562               | ON107281               | ON137583               |
|                    | FCZ40                         | ON137563               | ON107282               | ON137584               |
|                    | FCZ50                         | ON137564               | ON107283               | ON137585               |
| *F. globosum*      | CBS 428.97 T                  | KF46406                | KF464617               | MN534124               |
| *F. guttiforme*    | CBS 409.97 T                  | MT010967               | MT010999               | MT011048               |
| *F. konzum*        | CBS 119849 T                  | MW402733               | LT996098               | MN534095               |
| *F. lactis*        | CBS 411.97 ET                 | MN534275               | MN193862               | MN534077               |
| *F. longicornicola*| NRRL 52706 T                  | JF741114               | JF740788               | MW402360               |
| *F. denticulatum*  | CBS 407.97 T                  | MN534274               | MN534000               | MN534068               |
| *F. diminum*       | CBS 119860 T                  | KU171701               | MW401995               | MW402195               |
| *F. fujikuroi*     | CBS 221.76 T                  | KU604255               | MN534010               | MN534130               |
| *F. madaense*      | CBS 146669 T                  | MW402764               | MW402098               | MW402297               |
| *F. mangiferae*    | CBS 120994 T                  | MN534271               | MN534017               | MN534128               |
| *F. mexicanum*     | NRRL 53147 T                  | MN724973               | GU737282               | GU737494               |
| *F. mundagurra*    | RGB5717 T                     | KP083276               | KP083256               | MN534146               |
| *F. musae*         | CBS 624.87 T                  | MW402772               | FN552086               | FN545368               |
| *F. napiforme*     | CBS 748.97 T                  | MN534291               | MN193863               | MN534085               |
| *F. nygamai*       | CBS 749.97 T                  | EF470114               | MW402151               | MW402352               |
| *F. phyllophilum*  | CBS 216.76 T                  | KF466410               | MN193864               | KF466443               |
| *F. pilosicola*    | NRRL 29124 T                  | MN534248               | MN534055               | MN534099               |
| *F. proliferatum*  | CBS 480.96 ET                 | MN534272               | MN534059               | MN534129               |
| *F. pseudogammati* | CBS 417.97 T                  | MN534285               | AF160263               | MN534066               |
| *F. ramigenum*     | CBS 418.97 T                  | KF466412               | KF466423               | MN534145               |
| *F. sacchari*      | CBS 223.76 ET                 | JX171580               | MW402115               | MW402313               |
### Table 1. Cont.

| Species            | Culture Collection No./Isolate | GenBank Accession |
|--------------------|-------------------------------|-------------------|
|                    |                               | **rpb2** | **tef1** | **tub1** |
| F. siculi          | CBS 142222 T                  | LT746327 | LT746214 | LT746346 |
| F. succisae        | CBS 219.76 ET                 | MW402766 | AF160291 | U34419   |
| F. sudanense       | CBS 454.97 T                  | MN534278 | MN534037 | MN534073 |
| F. terricola       | CBS 483.94 T                  | LT996156 | MN534042 | MN534076 |
| F. thapsinum       | CBS 776.96 T                  | MN534289 | MN534044 | MN534080 |
| F. tijetauba       | NRRL 66243 T                  | KP083275 | KP083263 | GU737296 |
| F. tuiense         | NRRL 53984                    | LR792619 | GU737404 | GU737296 |
| F. prieskaense     | CBS 146498 T                  | MW834006 | MW834274 | MW834302 |
| F. foetens         | CBS 110286 T                  | MW928825 | MT011001 | MT011049 |
| F. hostae          | NRRL 29889 T                  | MT409446 | MT409456 | AM329042 |
| F. udum            | NRRL 25199 ET                 | KY498875 | KY498862 | KY498892 |
| F. stilboidea      | CBS 746.79 T                  | MW928832 | MW928843 |           |
|                    | FSY3                          | ON137577 | ON137556 | ON137598 |
|                    | FSY10                         | ON137578 | ON137557 | ON137599 |
|                    | FSY20                         | ON137579 | ON137558 | ON137600 |
| F. buharicum       | NRRL 25488                    | KX302928 | KX302912 |           |
| F. equiseti        | NRRL 26419                    | - | GQ505599 | -         |
|                    | NRR 20697                     | - | GQ505594 | -         |
|                    | NRRL 13405 T                  | GQ915491 | GQ915507 | GQ915441 |
|                    | DFS                           | - | MN823983 | -         |
| F. peruvianum      | CBS 511.75                    | - | MN120767 | -         |
| F. sarcochroum     | CBS 745.79                    | JX171586 | MW834278 | -         |
| F. inflexum        | NRRL 20433                    | JX171583 | AF084879 | U34435   |
| F. sublanatum      | NRRL 20897                    | KX302935 | KX302919 | -         |
| F. convolutans     | CBS 144207 T                  | LT996141 | LT996094 | -         |
| F. sarcochroum     | CBS 745.79                    | - | MW834278 | -         |
| Macroconia leptosphaeria | CBS 100001 | HQ728164 | KM231959 | KM232097 |

CBS: Westerdijk Fungal Biodiversity Institute (WIFB), Utrecht, The Netherlands. NRRL (Northern Regional Research Laboratory): Agricultural Research Service Culture Collection Database, Peoria, USA. CMW: The working collection of FABI (Forestry and Agricultural Biotechnology Institute), University of Pretoria, South Africa. BBA: Julius Kühn-Institute, Institute for Epidemiology and Pathogen Diagnostics, Berlin and Braunschweig, Germany. CPC: Collection of P.W. Crous. T: Ex-type specimen. NT: Neotype specimen. ET: Ex-epitype specimen. Accession numbers in **bold** belong to newly determined *Dendrobium officinale* isolates of *Fusarium* spp.

**2.3. Pathogenicity Studies**

To reproduce the dieback disease symptoms, the fungal isolates were tested for pathogenicity on the original host. A small, excised wound was accordingly made on the tip of each intact stem after being swabbed with ethanol 75% and washed with sterile water, then a mycelial agar disc (5 mm diameter) from each of the 7-day-old cultures of the fungal isolates was placed onto the surfaces of each stem tip and wrapped with Parafilm [8]. Afterward, the incubated plants were placed in a growth chamber at 25 °C and 75% relative humidity (RH) and maintained for 14 days. In contrast, the control plants received non-colonized agar plugs. Of note, the test was independently replicated thrice. All inoculated plants were visually assessed on a daily basis for up to two weeks. To fulfill Koch’s postulates, the same fungal isolates were re-isolated and their identity was confirmed by the *tef1* sequence data. To evaluate the disease severity, pear fruits (*Pyrus pyrifolia*) were also sterilized with 75% ethanol (3 min) and washed with sterile distilled water. Next, a mycelial agar disc (9 mm diameter) was placed on the fruit surface and covered with Parafilm to maintain high humidity [28]. All inoculated fruits were incubated under the same condition as mentioned above for one week. The fresh PDA agar plugs were further used as a negative control. The fruit rot diameter was finally measured by an electronic caliper seven days post inoculation (DPI). Each treatment included three replicates, and the experiment was independently repeated at least twice for both tests.
3. Results

3.1. Field Survey, Disease Symptoms, and Pathogen Isolations

In September 2021, symptoms of the dieback disease on D. officinale emerged in Yueqing, Zhejiang Province, China. According to the field observations, high temperature, high humidity, and poor ventilation would accelerate the incidence rate of this condition, which was at about 40–60% based on the number of plants with dieback disease symptoms recorded in 30 rows randomly picked. The symptoms appeared as chlorotic, blighted, and wilted leaves of the apical meristem with the shoot tip showing dark brown necrosis, dieback, and eventually shoot death (Figure 1). A total of 152 Fusarium-like isolates were also recovered from 70 infected plants, and 20 representative isolates were selected for further analysis (Table 2). Each isolate was recovered from different infected stems. Consistent with their morphological traits as well as molecular methods, the isolated fungi belonged to five genera, encompassing F. concentricum, F. curvatum, F. fujikuroi, F. nirenbergiae, and F. stilboides. Comparing the isolation frequency accordingly revealed that F. concentricum was the most abundant species, followed by F. curvatum, F. fujikuroi, and F. nirenbergiae, while F. stilboides was found the least (Table 2). Interestingly, two, and occasionally more than two, different Fusarium spp. were simultaneously isolated from some samples, and finally confirmed by the tef1 and rpb2 sequence analyses.

![Figure 1. Natural symptoms on Dendrobium officinale tissue associated with Fusarium spp. (A) Healthy plant; (B) typical dieback on the stem.](image)

| Geographic Origin            | Species        | No. Isolate | Isolation Frequency (%) |
|------------------------------|----------------|-------------|-------------------------|
| Zhejiang Province            | F. concentricum | 53          | 34.6                    |
| (Yueqing City)               | F. curvatum    | 34          | 22.3                    |
|                              | F. fujikuroi   | 28          | 18.4                    |
|                              | F. nirenbergiae | 21         | 13.8                    |
|                              | F. stilboides  | 16          | 10.5                    |
|                              | Total          | 152         | -                       |
3.2. Morphological Identification

The phenotypic criteria of the representative isolates obtained from the symptomatic stem tips matched with the descriptions of *F. concentricum*, *F. fujikuroi*, *F. nirenbergiae*, *F. curvatum*, and *F. stilboides* morphology.

In this regard, *F. concentricum* showed yellow-white, abundant, densely lanose to velutinous aerial hyphae with concentric rings on the PDA. Also, the colonies produced mainly 3-5-septate, naviculate to fusiform, slender macroconidia (Sporodochial conidia) with beaked apical and foot-shaped basal cells. Microconidia (aerial conidia) were also obovoid to fusoid, predominantly with no septa, but occasionally with 1 septum, and borne on mono or poly-phialides found in the aerial mycelia. However, chlamydospores were not observed. On the CLA, orange sporodochia were found (Figure 2).

![Figure 2. Morphological characteristics of Fusarium species isolated from infected Dendrobium officinale stems. (A) Front side on PDA; (B) Reverse side on PDA; (C) Sporodochia on Carnation Leaf Agar; (D) Macroconidia; (E) Microconidia. Scale bars: D–E = 10 µm.](image-url)

On the PDA, *F. curvatum* formed abundant floccose aerial mycelium with pale rosy white hue. Microconidia were also hyaline, ellipsoidal to falcate, 0-1-septate, and bore forming small false heads (i.e., short unbranched conidiophores) on the tips of the phialides. Besides, macroconidia were hyaline, 2-4-septate, banana-shaped, with blunt to papillate apical and blunt basal cells. Also, chlamydospores were not observed, and orange sporodochia formed on the carnation leave (Figure 2).

The *F. fujikuroi* colonies on the PDA consisted of floccose white aerial mycelia that became gray-violet or magenta with age, lacking chlamydospores. Notably, some swollen cells
could develop in the hyphae and superficially appear chlamydospores or pseudochlamydospores. Aerial conidia were also oval-shaped with a flattened base and 0-1-septate on the CLA. The long, slender, usually 3–6-septate macroconidia further proliferated on the monophialides of the branched conidiophores in the sporodochia. Moreover, the pale orange sporodochia was sparsely produced on the CLA (Figure 2).

The *F. nirenbergiae* colonies were pale vinaceous to burly-wood color, with abundant flocculent aerial hyphae on the PDA. Sporodochial conidia also formed small false heads on the tips of the phialides, lucid, oval to falcate with 0-1-septa. As well, macroconidia were hyaline, generally 3-septate, and in the shape of crescents or sickles with an attenuated to semi-papillate, curved apical and foot-shaped basal cells. The globose to spherical, aseptate chlamydospores were further produced terminally or intercalary. The aerial mycelium also formed abundantly bright orange sporodochia on the CLA (Figure 2).

The aerial mycelia of *F. stilboides* strains were cottony, velvety, reddish orange to maroon on the PDA. The aerial conidia were also long, cylindrical, smooth-walled, 3-5-septate, straight to almost slightly flexuous in the center and sharpened at the apices with marked foot-shaped cells. Moreover, formation of orange sporodochia was observed on CLA. The microconidia were typically obovoid to elliptical and 0-1-septate, and chlamydospores were present (Figure 2).

### 3.3. Molecular Identification and Phylogenetic Analyses

The PCR amplified partial sequences of the genes *tef1*, *rpb2*, and *tub2*, yielded 651, 718, and 481 bp fragments, respectively. The BLASTN searches against all *Fusarium* sequences in the GenBank additionally showed that the 20 representative isolates included in this study shared 99–100% similarity with type-strains of five *Fusarium* spp., namely, *F. concentricum*, *F. fujikuroi*, *F. nirenbergiae*, *F. curvatum*, and *F. stilboides*, which supported previous efforts for the identification of these pathogens based on the macro and micro-morphological characteristics. To clarify the phylogenetic relations, the phylogenetic trees were built here from the single genes *tef1* and *rpb2*. These trees included several sequences from the new isolates, *Fusarium* type strains, plus a few non-type strains. The given trees further supported the identification of the *D. officinale* isolates (Supplementary Figure S1). The partial *tub2* sequences also displayed close similarity with the *Fusarium* species but provided an insufficient resolution to identify them. For further molecular verification, multilocus phylogenetic analysis (MLSA) was further performed based on 1850 nucleotide positions among 91 in-group taxa, including clades corresponding to FOSC, FFSC, FLSC, and FIESC. The MLSA tree accordingly indicated that the *D. officinale* isolates in the present study clustered unambiguously with *F. concentricum*, *F. fujikuroi*, *F. nirenbergiae*, *F. curvatum*, and *F. stilboides* type strains with the bootstrap values of 99%, 99%, 96%, 95%, and 99%, respectively (Figure 3). The topologies of the trees obtained from each individual gene also resembled each other and were, above all, similar to the MLSA tree (Supplementary Figure S1). Nonetheless, the concatenated dataset, in addition to all the individual phylogenies, clearly determined the phylogenetic relationship and taxonomy of Tiepishihu isolates in the Fusarioid taxa. Moreover, the *F. equiseti* strain DFS, as a pathogen of the dieback disease of *D. officinale* in China, fell strongly with the *F. equiseti* clade, which belonged to the FIESC (Supplementary Figure S1).

### 3.4. Pathogenicity Assays

Two weeks after inoculation, the pathogenicity test results revealed that the isolates from five *Fusarium* spp. had the typical black brown necrosis and the dieback symptoms on the tip of *D. officinale* (tie-pie variety) stems, which were congruent with the field observations, while no symptoms developed on the control plants inoculated with the agar media. All these fungal species were also re-isolated from the inoculated plants, and identified using the *tef1* locus, thereby fulfilling Koch’s postulates (Figure 4A). Notably, the dieback disease symptoms incited by each pathogen were indistinguishable in the field. As such, all *Fusarium* spp. isolates were pathogenic on Tiepishihu and caused the
Furthermore, *F. concentricum* and *F. fujikuroi*, among the assayed isolates, indicated higher virulence on pears than the others, followed by *F. nirenbergiae* and *F. curvatum*, which had relatively similar disease severity, whereas *F. stilboides* showed the lowest virulence in this respect (Figure 4B; Table 3).

**Figure 3.** Multilocus phylogenetic tree resulting from maximum likelihood analysis of concatenated rpb2, tef1, and tub2 sequences. The tree shows the phylogenetic relationships of *Fusarium* spp. causing dieback disease in *Dendrobium officinale*. Isolates recovered from Tiepishihu in this study are indicated by a black circle (●). Clades including isolates obtained from *D. officinale* are shaded in color. The tree is rooted to *Macroconia leptosphaeria* CBS 100001. Bootstrap values are shown above the branches. Subdivision of the *Fusarium* clade represents the recognized species complexes.
which was regarded as a FOSC member. However, little is known about this species, *Podocarpus macrophyllus* wilt on *Fusarium* The study findings also suggested that the pathogen on various host plants including cotton, *Fusarium* pathogens and innocuous saprophytes, e.g., *Fusarium* Table 3. Difference test (LSD). letters represent significantly different values at 7 DPI in two perpendicular directions. Data are mean ± SE. The mean values followed by different letters represent significantly different values at *p* < 0.05 among species using the least significant difference test (LSD).

4. Discussion

The dieback disease has already plagued the *Dendrobium officinale* industry with a high incidence rate in Zhejiang Province, China. On the basis of the MLSA supported by morphological observations in this study, five distinct taxa, viz., *F. concentricum*, *F. fujikuroi*, *F. nirenbergiae*, *F. curvatum*, and *F. stilboides*, causing the dieback disease on Tiepishihu, were diagnosed. The study findings also suggested that the *Fusarium* species associated with this condition on Tiepishihu were more diverse than the ones previously recorded [8]. The Koch’s postulates corresponding showed that the *Fusarium* spp. Isolates were infective in nature, with slight variations in virulence.

The newly inflicted *Fusarium* spp. On *Dendrobium officinale* have been also found as plant pathogens and innocuous saprophytes, e.g., *F. concentricum* causing fruit blotch on *Hibiscus sabdariffa*, stem rot on *Paris polyphylla*, fruit rot on pepper and banana, leaf spot on mango, wilt on *Podocarpus macrophyllus*, and ear rot on maize, originally named by Nirenberg and O’Donnell [24,29–32]. *F. curvatum* has been also described as a new taxon by Lombard et al. [16], originally named *F. oxysporum* (*matthiolae* and *meniscoideum formae speciales*), which was regarded as a FOSC member. However, little is known about this species, and it has been reported as a pathogen on yam [13,16,24,33]. In addition, *F. fujikuroi* is a well-studied taxon with sexual stage, *Gibberella fujikuroi* (Sawada), which is known as a pathogen on various host plants including cotton, *Echinochloa* sp., grapes, maize, *Macleaya cordata*, *Reineckia carnea*, rice, soybean, strawberries, sugarcane, wheat, and *Zanthoxylum armatum* [24,32,34]. Moreover, *F. nirenbergiae* was recently resolved from the FOSC and included several *formae speciales* of *F. oxysporum* (viz., *dianthi*, *chrysantheni*, *bouvardiae*, *adices-lycopersici*, *cubense*, *lycopersici*, and *passiflorae*) and clinically relevant strains [13,16,24]. This
pathogen has been further associated with multiple diseases such as saffron corm rot, wilt on Acer negundo, Dipladenia sp., and passion fruit [16,35–37]. Fusarium stilboides is an FLSC member that has been recorded as a pathogen on bamboo, Capsicum annuum, carnation, coffee, and passion fruit [13,38–41].

Although macro- and micro-morphological observations alone may be insufficient, several critical characteristics can provide useful information for discriminating the Fusarium species [13–16]. Hence, the detailed and close morphological examinations form an important part in the classification of this genus. In this line, F. curvatum produces high curvature macroconidia and aerial polyphialidic conidiogenous cells, differentiating it from F. nirenbergiae and other species [13,14,16]. Moreover, chlamydospores are readily present in F. nirenbergiae, while this feature is not known for F. concentricum, F. fujikuroi, and F. curvatum [16–24]. However, F. stilboides can produce chlamydospores even though this trait is not taxonomically useful [24]. Additionally, F. nirenbergiae cannot form polyphialides, and resembles F. oxysporum in this respect [16–24]. Morphologically, both F. fujikuroi and F. concentricum proliferate microconidia in false heads or chains, whereas the conidiophores of the aerial mycelium are only sparsely branched in F. concentricum with only a few polyphialides, and conidia in F. fujikuroi conidia are from polyphialides, and less often from monophialides [24,30,32].

The validity of morphological identification in this study was thus confirmed by the phylogenetic analyses derived from the molecular results. The tef1, rpb2, and tub2 genetic barcodes were thus selected for this purpose because they consisted of phylogenetically informative sequences for the differentiation and classification of Fusarium species [13,16,19,20].

The tef1 phylogeny accordingly demonstrated better resolution at the species level in comparison with rpb2 and tub2. For the concatenated gene analyses, the topologies of the trees inferred for individual genes were also evaluated visually to establish that the overall tree topology of the single locus datasets were similar to each other and to that of the tree acquired from the combined dataset alignment. In the MLSA tree, the Fusarium isolates from D. officinale in the present study were phylogenetically different from each other and were situated within the FOsc, FFSC, and FLSC clades with F. concentricum CBS 450.97, F. curvatum CBS 238.94, F. fujikuroi CBS 221.76, F. nirenbergiae CBS 840.88, and F. stilboides CBS 746.79 type strains, while none of them fell in the FSbc, Fsc, and FsaSc clades. Even if F. nirenbergiae and F. curvatum were placed in the same clade, they formed two distinct well-supported subclades, which correlated with clade VIII resolved by Lombard et al. [16]. Furthermore, the tree topology of the concatenated dataset in this study was closely similar to the trees developed by Lombard et al. [16], Crous et al. [13], and Yilmaz et al. [19].

Interestingly, it has been concluded that the dieback is a disease complex, induced by one or more Fusarium spp. (viz., F. concentricum, F. curvatum, F. fujikuroi, F. nirenbergiae, and F. stilboideae), as observed in manifold crops [33,42–44]. For instance, eight species including F. asiaticum, F. equiseti, F. fujikuroi, F. graminearum, F. meridionale, F. oxysporum, F. proliferatum, and F. verticillioides, have been evidenced to incite corn sheath rot in Sichuan Province, China [44]. Similarly, ten Fusarium species, viz., F. asiaticum, F. commune, F. cugenangense, F. curvatum, F. fujikuroi, F. gossypinum, F. nirenbergiae, F. odoratissimum, F. solani and F. verticillioides, have been detected to cause wilt on yam [33].

Despite much effort, there was no success in defining the pathogen-specific diagnostic criteria for these five taxa, which induced the dieback disease on D. officinale. Further surveys are thus required to establish the species-specific symptoms of this condition. Additionally, the symptoms on some stem tips may slightly differ in respect of intensity or color, suggesting that such symptoms may have been due to secondary infections by saprophytic microbes or affected by environmental conditions, such as high humidity or unfavorable ventilation, as documented in previous research [21,22,35,45–50].
5. Conclusions

In sum, the study data here confirmed that the losses in *D. officinale* yields were caused by *F. concentricum*, *F. curvatum*, *F. fujikuroi*, *F. nirenbergiae*, and *F. stilboides*. Notably, the incidence rate of these local outbreaks could be triggered by environmental factors, and it is expected to increase in the future because of both climate change and susceptible cultivars. Regarding the significance of this study, it provided information on the biodiversity and epidemiology of *Fusarium* spp. associated with the dieback disease, which can contribute to the development of breeding programs and disease management strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof8090919/s1. Figure S1: Phylogenetic tree of the *Fusarium* species resulting from maximum likelihood based on the sequence of *tef1* gene. Bootstrap support values were given at the nodes. Isolates isolated in this study are shown by a black circle (●), while *F. equiseti* strain DFS is indicated by a black square (■).

Author Contributions: Conceptualization, design of the experiments, methodology, bioinformatic analysis, interpretation of the data, and writing the original manuscript, S.A.M.; editing the manuscript, T.M.; advising on the experiments and providing resources, W.C.; validation, funding acquisition, supervision, B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Key Research and Development Projects in Zhejiang Province (No. 2018C02034).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequences have been deposited in GenBank (Table 1). The data presented in this study are openly available in NCBI. Publicly available datasets were analyzed in this study. These data can be found here: https://www.ncbi.nlm.nih.gov/ (accessed on 2 April 2022).

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

References

1. Nie, S.; Cui, S.W.; Xie, M. Glucomannans from *Dendrobium officinale* and aloe. *Bio. Polym.* **2018**, *6*, 295–347. [CrossRef]
2. Cheng, J.; Dang, P.P.; Zhao, Z.; Yuan, L.C.; Zhou, Z.H.; Wolf, D. An assessment of the Chinese medicinal *Dendrobium* industry: Supply, demand and sustainability. *J. Ethnopharmac.* **2019**, *229*, 81–88. [CrossRef] [PubMed]
3. Sarsaiya, S.; Jain, A.; Jia, Q.; Fan, X.; Shu, F.; Chen, Z. Molecular identification of endophytic fungi and their pathogenicity evaluation against *Dendrobium nobile* and *Dendrobium officinale*. *Int. J. Mol. Sci.* **2020**, *21*, 316. [CrossRef]
4. Shan, T.; Zhou, L.; Li, B.; Chen, X.; Guo, S.; Wang, A. The plant growth-promoting fungus MF23 (*Mycoena* sp.) increases production of *Dendrobium officinale* (*Orchidaceae*) by affecting nitrogen uptake and NH4⁺ assimilation. *Front. Plant Sci.* **2021**, *12*, 693561. [CrossRef] [PubMed]
5. Teoh, E.S. Dwelling on rocks (Shihu). In *Orchids as Aphrodisiac, Medicine or Food*; Springer: Berlin/Heidelberg, Germany, 2019; Volume 4, pp. 69–86. [CrossRef]
6. Zhang, Y.Q.; Lin, B.Y.; Zou, M.Y.; Liang, J.X.; Hu, H.Q. First report of Fusarium wilt of *Dendrobium officinale* caused by *Fusarium oxysporum* in China. *Plant Dis.* **2017**, *101*, 1039. [CrossRef]
7. Xie, Y.Y.; Wang, L.P.; Fang, L.; Wang, H.R. First report of leaf spot caused by *Phoma multistrostrata var. microspora* on *Dendrobium officinale* in Zhejiang Province of China. *Plant Dis.* **2018**, *102*, 1655. [CrossRef]
8. Guo, M.; Li, B.; Wang, R.; Liu, P.; Chen, Q. Occurrence of dieback disease caused by *Fusarium equiseti* on *Dendrobium officinale* in China. *Crop Prot.* **2020**, *137*, 105209. [CrossRef]
9. Xiao, C.; Li, R.; Song, X.; Tian, X.; Zhao, Q. First report of soft rot on *Dendrobium officinale* caused by *Epicoccum sorghinum* in China. *Plant Dis.* **2021**, *1063*. [CrossRef]
10. Xiao, C.; Li, R. Detection and control of *Fusarium oxysporum* from soft rot in *Dendrobium officinale* by Loop-Mediated isothermal amplification assays. *Biology* **2021**, *10*, 1136. [CrossRef]
11. Cao, P.; Zheng, Z.; Fang, Y.; Han, X.; Zou, H.; Yan, X. First report of stem rot caused by *Fusarium kyushuense* on *Dendrobium officinale* in China. *Plant Dis.* **2022**, *31*, 2257. [CrossRef]
12. Cao, P.; Zheng, Z.; Han, X.; Zou, H.; Yan, X. Occurrence of Neopestalotiopsis clavispora causing leaf spot on *Dendrobium officinale* in China. *Plant Dis.* **2022**, *12*, 1761. [CrossRef] [PubMed]
13. Crous, P.W.; Lombard, L.; Sandoval-Denis, M.; Seifert, K.A.; Schroers, H.J.; Chaverri, P.; Gené, J.; Guarro, J.; Hirooka, Y.; Bensch, K.; et al. *Fusarium*: More than a node or a foot-shaped basal cell. *Stud. Mycol.* **2021**, *98*, 100116. [CrossRef] [PubMed]
42. López-Moral, A.; Lovera, M.; Raya, M.D.C.; Cortés-Cosano, N.; Arquero, O.; Trapero, A.; Agustí-Brisach, C. Etiology of branch bieback and shoot blight of english walnut caused by Botryosphaeriaceae and Diaporthe species in southern Spain. Plant Dis. 2020, 104, 533–550. [CrossRef]

43. Guo, Z.; Li, Q.; Tang, L.; Guo, T.; Huang, S.; Mo, J.; Hsiang, T.; Luo, S. Fusarium species associated with leaf spots of mango in China. Microb. Pathog. 2021, 150, 104736. [CrossRef]

44. Wang, W.; Wang, B.; Sun, X.; Qi, X.; Zhao, C.; Chang, X. Symptoms and pathogens diversity of corn Fusarium sheath rot in Sichuan Province, China. Sci. Rep. 2021, 11, 2835. [CrossRef]

45. Yu, J.; Babadoost, M. Occurrence of Fusarium commune and F. oxysporum in horseradish roots. Plant Dis. 2013, 97, 453–460. [CrossRef] [PubMed]

46. Asselin, J.; Bonasera, J.M.; Beer, S.V. PCR primers for detection of Pantoea ananatis, Burkholderia spp., and Enterobacter sp. from onion. Plant Dis. 2016, 100, 836–846. [CrossRef] [PubMed]

47. Swett, C.L.; Science, P.; Architecture, L.; Park, C.; Sharon, C. Evidence for a hemibiotrophic association of the pitch canker pathogen Fusarium circinatum with Pinus radiata. Plant Dis. 2016, 100, 79–84. [CrossRef] [PubMed]

48. Degani, O.; Movshovitz, D.; Dor, S.; Meerson, A.; Goldblat, Y.; Rabinovitz, O. Evaluating azoxystrobin seed coating against maize late wilt disease using a sensitive qPCR-based method. Plant Dis. 2019, 103, 238–248. [CrossRef]

49. Mayorquin, J.S.; Nouri, M.T.; Peacock, B.B.; Trouillas, F.P.; Douhan, G.W.; Kalsen, C. Identification, pathogenicity, and spore trapping of Colletotrichum karstii associated with twig and shoot dieback in California. Plant Dis. 2019, 103, 1464–1473. [CrossRef] [PubMed]

50. Bock, C.H.; Barbedo, J.G.A.; Del Ponte, E.M.; Bohnenkamp, D.; Mahlein, A.K. From visual estimates to fully automated sensor-based measurements of plant disease severity: Status and challenges for improving accuracy. Phytopathol. Res. 2020, 2, 9. [CrossRef]