Pathogenicity of fungus strains isolated from medicinal *Fritillaria przewalskii* Maxim. bulb rot

rui Wu (415015184@qq.com)
Gansu Agricultural University https://orcid.org/0000-0002-1026-2480
Yuan Chen
Gansu Agricultural University
Fengxia Guo
Gansu Agricultural University
Yuan Gao
Gansu Agricultural University
Linlin Dong
China Academy of Chinese Medical Sciences

Research

Keywords: Fritillaria przewalskii Maxim., bulb rot pathogens, isolation and identification, pathogenicity

Posted Date: April 3rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-20309/v1

Abstract

Background: Bulb rot causes loss of the perennial medicinal plant *Fritillaria przewalskii* Maxim., is exacerbated with growth years and seriously constrains plant productivity, but the pathogens responsible for the disease were still unknown, very few reports were presented before.

Methods: To determine potential pathogenic sources, fungus strains were isolated from diseased plant samples with bulb rot symptom of the 3- to 5-year-old *F. przewalskii* plants by tissue separation, and assayed for pathogenicity according to Koch's Law.

Results: Seven pathogenic strains (F1-F7) were detected in5-year-old rot bulbs, six (F1-F6) in 4-year-old rot bulbs, and four (F1, F2, F5, F6) in 3-year-old rot bulbs. All of the strains were able to infect bulbs by stabbing and some exhibited varying levels of aggressiveness. Relative to the non-stabbing controls, the bulbs stab-inoculated with F5, F2, F7, F4, F1, F6, and F3 showed 76.65%, 75.15%, 71.44%, 40.37%, 39.09%, 36.87%, and 34.93% rot after 8 days, respectively. Phylogenetic analysis revealed that theses trains (deposited in GenBank under accessions MH917682 to MH917688) were clustered into *Bionectria ochroleuca* (F1, F3, F4), *Fusarium oxysporum* (F2, F7), *Fusarium tricinctum* (F5), and *Clonostachys rosea* (F6). The two species of *Fusarium* had the strongest pathogenicity, followed by *Bionectria ochroleuca* and *Clonostachys rosea*. Although leading to low bulb rot incidence by stab-inoculation, F1 showed the highest isolation rate (48.9%) among all strains.

Conclusions: Thus, the edible and medicinal bulbs of *F. przewalskii* are susceptible to synergetic contamination by these seven pathogenic strains at some point after their third year of growth, which has contributed to the species endangered status, with the two strains of *Fusarium* being the predominant pathogens. To our knowledge, this is the first report on the seven strains of four fungal species causing in *F. przewalskii* bulb rot in China.

Background

Fritillaria przewalskii Maxim. is a perennial herbaceous plant in the Liliaceae family. Bulbus Fritillariae, which refers to the dry bulbs of F. przewalskii, F. cirrhasa, F. unibracteata, F. delavayi, and F. taipaiensis, have been used as antitussive, anti-asthmatic, expectorant, and antihypertensive drugs in traditional Chinese medicine under the name "Chuanbeimu" for thousands of years [1]. Bulbus F. Cirrhosae contain alkaloids and saponins, and the content of the latteris higher than that of other 'Beimu' [2]. The bulbs have unique pharmacological effects on tuberculosis cough, spitting hemoptysis, heart stagnation, and cancer [3]. Therefore, they are extensively used as a therapeutic agent in China. Wild F. przewalskii are indigenous to the alpine regions of central Maqu County, southwest to Tibetan Autonomous Prefecture, but can also be found in the Zhang, Min, and Weiyuan counties of Gansu Province, China [4], being commonly known as 'Minbei'. The plant lives in cold, humid meadows and brushy high-altitude steppes. However, they are very rare in southern Qinghai, eastern Tibet, and western Sichuan in China [5].

The demand for ‘Minbei’ has greatly increased with health care awareness and therefore its wild sources have been severely depleted. The over exploitation of this resource, coupled with its fragile ecosystem, caused F. przewalskii to disappear quickly. As a result, it now appears on the list of Chinese endangered wild protected plants [6]. Its scarcity has driven up the costs of the medicinal materials derived from it far above those obtained from other Fritillaria species [3]. This plant usually requires 4 to 5 years from seed germination to blossoming [4] and, although perennial, the annual spring time greening period of its aboveground part lasts for only 60 to 90 days. In contrast, its underground bulbs and rhizomes take several years to develop [6]. During this long growth period, the bulbs are susceptible to rot, which substantially reduces their active ingredients and yield.

Root rot is caused by various pathogens and it is accelerated by high temperature, humidity, and high planting density [7]. Mycelia or spores present in soil or diseased plant residues enter host plant wounds and initiate infection. Wounds caused by insects may be the main entry points of root rot pathogens [8, 9], the types of which vary with plant species and habitat. Furthermore, pathogens may behave differently in the same plant species growing in different habitats. For instance, Fusarium sp. was the dominant pathogen causing berry root rot in Ningxia and Qinghai Provinces [10] but Phytophthora sp. and Rhizoctonia solani caused wolfberry root rot in Xinjiang Province [11]. Fusarium oxysporum and Fusarium solani were the predominant pathogens of Lycium bararum in Gansu Province [12]. Whereas in Weiyuan county, Fusarium oxysporum, Fusarium solani, and Rhizoctonia solani were primarily responsible for medicinal Astragalus membranaceus root rot [9], only Fusarium solani and Fusarium oxysporum caused this disease in Longxi County of Gansu Province [13]. On the other hand, Fusarium solani alone induced Astragalus sp. root rot in a Shanxi Province production area [14]. However, Fusarium solani, Fusarium oxysporum, and Fusarium moniliforme infected Astragalussp. roots in the Ningxia Hui Autonomous Region [15]. Bulbous Fritillaria sp. root rot was shown to be caused by the blue variants of F. solani var. coeruleum in Gongliu, Yili of Xinjiang, China [16], while F. hupehensis (‘Hubeibeimu’) root rot was caused mainly by Fusarium oxysporum [17].

The abovementioned studies provide critical information that can be used to protect medicinal plants from fungal root rot. Nevertheless, the ability of an endangered medicinal plant to resist root rot may be associated with its environmental stress tolerance. To our knowledge, previous studies investigating bulb rot in endangered medicinal plants have not attempted to identify the pathogens involved. In this research, we focused on identifying the fungal strains responsible for bulb rot in a rare alpine medicinal plant as well as their modes of action. Fungal isolates were extracted from diseased bulbs growing for 3 to 5 years in a wild fostered field. After incubation for 8 days, the isolates were identified according to their morphology and using molecular biology techniques. The general pathogenesis of bulb rot was elucidated for this endangered medicinal plant. This information may be used to protect this resource and to ensure its sustainable cultivation in the future.

Materials And Methods

Artificial wild fostering of F. przewalskii

F. przewalskii plants were artificially fostered on wild meadow soils along a 15° mountain slope at 2,700 m altitude in Zhang County, Gansu Province, China (34°35'29" N,104°19'41" E). The area showed 8.6℃ average annual temperature, 126 frost-free days on average, and 500 mm mean annual rainfall. The experimental field was located on the 2,500 m^2 upper area of the wild domestic field fostered by Chen Yuan from Gansu Agricultural University.

Capsules were collected from wild healthy plants in the same period during 2012–2014 and at the same locality within the traditional geo-authentic-producing areas of Zhang County at 2,927 m altitude (34°51'19" N, 104°01'05" E). After carefully wisped in stems, the capsules were transported within 6 h to the experimental site, inside woven bags. The seeds were carefully stripped out of capsules into a basin, covered with moss crusts, and immediately prepared for sowing. The mean 1000-seed fresh weight was 1.610 ± 0.194 g (mean ± Stand Error (SE), n = 10).

The fostered experiment was conducted in a single factor random block design, in which sowing year had three levels (2012, 2013, 2014) with 10 replicates each. Wild plant seeds were sown on August 2, 2012 and on the same day in 2013 and 2014. Before sowing, the field was divided into 30 long-ridge beds (1 m width × 10 m length, 5 cm height) along the slope gradient. The seeds were then sown by furrowing on the beds, at 3 cm depth, and at 10 cm from each other, resulted in 8.0 g/m^2 sowing density; 10 soil ridge beds were sown each year. The distance between each two ridges was 0.3 m and the total experimental field occupied approximately 390 m^2. Fertilization, weeding, detailed observation, and records were maintained after sowing.

Harvest of fresh F. przewalskii bulbs

At the optimal harvest time for the medicinal materials of F. przewalskii (July 20, 2017), samples of healthy and diseased bulbs and their respective rhizosphere soils were collected, placed in zip-lock plastic bags, labeled, stored in a portablethermal-insulationboxwith ice, and transported to laboratory within 12 h after sampling where they were stored at 4 °C until analyses.

Determination of F. przewalskii bulb characteristics

Twenty bulbs were randomly sampled from both healthy and diseased plants 3 to 5 years old. Their color was recorded and characteristics such as diameter, length, and fresh weight were measured with an electronic Vernier caliper and an analytical balance (1:10,000).

Isolation and purification of the F. przewalskii bulb rot pathogens

Diseased F. przewalskii bulb samples were excised from the bulbs and cut into blocks (3–4 mm^2) along the lesions observed on tissues. For the surface sterilization and isolation of rot pathogens, we followed recently improved and well established methods [8, 9]. The sample blocks were thoroughly washed in running tap water for 30 min and rinsed with double-distilled water for 10 min, before being surface sterilized with 75% ethanol for 5 s, 0.1% (w/v) mercury chloride for 30 s, rinsed four times with sterile

double-distilled water, and finally dried on sterile filter paper under aseptic conditions. Three dried bulb blocks from each age group were inoculated onto a culture dish (90 mm × 90 mm) containing potato dextrose agar (PDA) medium consisting of 200 g L^{-1} potato, 16 g L^{-1}dextrose, and 16 g L^{-1} agar. Fifteen replicates were used, and the incubation period was 3 to 4 days at 25 ± 1 °C in the dark. Finally, and based on the colony morphology of isolates [8, 18], the isolate separation frequency (ISF) was calculated as ISF (%) = (In / TB) × 100, where 'In' is the number of isolates with the same morphology and 'TB' is the total bulb blocks in each culture dish for each growing-year bulb group [19].

All the above isolates were purified on fresh PDA medium by the plate line dilution separation method [8]. They were stored on slant PDA culture medium at 4 °C until used for the pathogenicity tests [20].

Pathogenicity Tests

The fungal strains were tested for pathogenicity on water agar (WA) medium containing 7 g L^{-1} dextrose. Three healthy bulbs from the above samples stored at 4 °C in each growing-year group were punctured and inoculated with one cake of isolates (5 mm in diameter) in WA medium, and incubated in the dark for 8 days according to Koch's Law [21]. Punctured and non-punctured healthy bulbs inoculated only with PDA medium were used as controls. The pathogenicity tests were repeated six times for all the 3- to 5-year-old bulbs. The culture conditions for the pathogenicity tests were the same as those used in the isolation process. The incidence of pathogens (isolated from the diseased bulbs) in healthy bulbs was calculated from the ratio of total infected bulbs (If) to total inoculated bulbs (In), i.e., Incidence rate (%)= (If /In) × 100. Pathogens from infected bulbs with typical rot symptoms were isolated and identified by Koch's Law to confirm that they were indeed the same pathogens causing bulb rot.

Morphological observation of the pathogens causing F. przewalskii bulb rot

The pathogens isolated from diseased F. przewalskii bulbs were inoculated on PDA medium and incubated under thermostatic conditions (25 ± 1)°C for 35 days in the dark. Colony and conidium morphology were used for identification [18]. Colony morphology was first observed with naked eye for macroconidia and chlamydospore formation and then under a scanning electron microscope (SEM; BX61; Olympus, Tokyo, Japan).

Molecular identification and phylogenetic analysis of the pathogens causing F. przewalskii bulb rot

Mycelia were collected from all 7-day-old isolates cultured on PDA medium and ground in liquid nitrogen with a disposable pestle. Total deoxyribonucleic acid (DNA) was then extracted using an E.Z.N.A.™HP Fungal DNA Kit according to the manufacturer's instructions (Omega, Chengdu, China). DNA concentration and quality were evaluated with a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular Identification

The internal transcribed spacer (ITS) region of the fungal pathogens DNA was amplified with the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [22] in a Bio-Rad T-100™ Thermal Cycler Polymerase Chain Reaction (PCR) (186–1096; Bio-Rad Laboratories, Hercules, CA, USA). The PCR amplification was performed as described in Tamuraet al. [23] with slight modifications. The final reaction volume (25 μL) contained 1 μL template genomic DNA (~ 100 μg), 1 μL each primer (10 μM), and 12.5 μL Taq PCR Mastermix (Sangon Biotech, Shanghai, China). The PCR amplification conditions were set as follows: 3 min incubation at 94 °C; 37 cycles of 0.5 min at 94 °C, 1 min at 55 °C, 1 min at 70 °C; and a final extension of 10 min at 72 °C. The PCR products were separated on a 1% agarose gel, stained, and photographed with a gel imaging system. Successful PCR reactions with specific bands were sent out for sequencing (Sangon Biotech, Shanghai, China). All DNA sequences originally obtained for the seven strains were submitted to GenBank using the Sequin Program (National Center for Biotechnology Information, NCBI).

The sequences of the fungal strains originally isolated from diseased F. przewalskii bulbs were aligned with those in the GenBank database via the basic local alignment search tool (BLAST) with default settings. Pairwise alignments among highly similar accessions were compared using the Needleman-Wunsch Global Alignment tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.Cgi). Sequences with high similarity were used to construct a phylogenetic tree of related strains in MEGA version 5.0 [23]

Statistical analysis

Descriptive statistics of quantitative data were performed to compare the characteristics of healthy and diseased bulbs. Statistical analyses were performed using Excel 2007 (Microsoft, Redmond, WA, USA) and SPSS, Version 11.5 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SE and percentage. Differences between healthy and diseased plants in the same growing-year were analyzed for statistical significance with the two-tailed t-test. Variance was analyzed using the Duncan's multiple comparison method considering plant type (two levels: healthy and diseased), bulb growing-year (three levels: 3, 4, 5 growing-year groups), and their combinations, as well as the frequencies of the seven isolated strains, as factors. Significant differences were defined at $P \leq 0.05$ and $P \leq 0.01$.

Results

Field symptoms of bulb rot in infected F. przewalskii plants

Healthy and diseased F. przewalskii plants grown on a wild fostered field in Zhang County, Gansu Province, China, are shown in Fig. 1. Pathogens infected both the bulbs and the roots, adhering to them and causing partial or entire organ tissue discoloration and decay; these symptoms were more obvious in plants older than 3 years. Tissue destruction in the diseased roots hindered nutrient and water translocation to the shoots leading to plant wilt and, ultimately, death (Fig. 1). However, these latter symptoms were seldom observed in 1- or 2-year-old plants. Diseased plants older than 3 years showed diverse morphologies, and only a few exhibited root symptoms but not shoot and bulb symptoms (Fig. 1d). The infected bulbs were inlayed with yellow-brown, black-brown, or black-green spots on their

surfaces (Fig. 1e, h). The lesions were absent in healthy bulbs (Fig. 1f, g, i). As the disease spread, the bulbs decayed, preventing water and nutrient transport from the soil to the aboveground plant parts. Mildly infected plants wilted in the midday sun and recovered at nighttime while severely infected plants died after sun exposure

Bulb diameter, height, and fresh weight were measured to correlate bulb size and rot incidenceto growing-year classification (Fig. 1j, k, l). Bulbs were larger in fifth > fourth > third growing-year (Fig. 1j, k, l) and variance analysis revealed significant differences in bulb diameter ($F = 328.632$, $P < 0.01$), height ($F = 177.746$, $P < 0.01$), and fresh weight ($F = 349.182$, $P < 0.01$) among the three growing-years in both healthy and diseased plants (d.f. = 2, 95). Healthy bulbs' diameter ($F = 9.230$, $P < 0.01$) and fresh weight ($F = 10.670$, $P < 0.01$) were significantly higher than those of diseased bulbs, on average (d.f. = 1, 95), but this was not observed for bulb height. A significant interaction between growing-year and plant type (diseased or healthy) was only found in for bulb diameter ($F = 3.553$, d.f. = 2.95, $P < 0.05$). No significant differences in bulb height and diameter were detected between diseased and healthy bulbs in the same growing-year according to the t-test (n = 20).

Pathogen Isolation And External Colony Morphology

Fungal pathogens were isolated from diseased 3- to 5-year-old F. przewalskii bulbs and incubated in PDA medium in the dark for 4 days (n = 45; Fig. 2). The fungal isolates varied with bulb age and seven fungal strains (F1-F7) were isolated from the diseased bulbs. All seven strains were identified in the 5-year-old bulbs (Fig. 2a), F1-F6 were found in the 4-year-old bulbs (Fig. 2b), and only F1, F2, F5, and F6 were present in the 3-year-old bulbs (Fig. 2c). Certain blocks from the inoculated bulbs had multiple isolates whereas others had only one and a few had none. The bulb growing-year had no significant effect on the separation frequency of these isolates ($F = 0.653$, d.f. = 2,42, $P = 0.526$). However, bulb age was strongly correlated with the isolates themselves ($F = 57.084$, d.f. = 6,42, $P < 0.01$), and a significant interaction between growing-year and the isolates was also found ($F = 2.552$, d.f. = 12.42, $P < 0.05$). Isolates' separation frequencies were ranked as F1 > F2 > F5 > F4 > F6 > F3 > F7. At $\leq 50\%$, F1 showed the highest isolation frequency which was greater than those of F2-F7, and it increased by 53.5% ($t = 3.080$, $P < 0.01$, n = 45), 3,200.0% ($t = 10.742$, $P < 0.01$, n = 45), 407.7% ($t = 6.664$, $P < 0.01$, n = 45), 127.6% ($t = 5.692$, $P < 0.01$, n = 45), 1,550.0% ($t = 10.298$, $P < 0.01$, n = 45), and 6,500.0% ($t = 11.026$, $P < 0.01$, n = 45), respectively (Table 1). F2 and F5 had the second and third highest isolation frequencies. These were significantly lower than that of F1 but significantly higher than those of the other four strains. The isolation frequencies of F3, F4, F6, and F7 were all < 10.0% and there were no significant differences among them.

Table 1
Isolated frequency of the strains from 3–5 growing years of Fritillaria przewalskii rot bulbs

Species	Strain codes	Isolated frequency (%)			
		3 years	4 years	5 years	Mean
Bionectria ochroleuca	F1	46.7 ± 3.9	60.0 ± 6.7	40.0 ± 7.7	48.9 ± 4.3^{aA}
	F3	0	2.2 ± 2.2	2.2 ± 2.2	1.5 ± 1.0^{eD}
	F4	0	8.9 ± 5.9	20.0 ± 7.7	9.6 ± 4.0^{dD}
Clonostachys rosea	F6	4.4 ± 2.2	2.2 ± 2.2	2.2 ± 2.2	3.0 ± 1.2^{deD}
Fusarium tricinctum	F5	24.4 ± 2.2	22.2 ± 5.9	17.8 ± 2.2	21.5 ± 2.2^{cC}
Fusarium oxysporum	F2	40.0 ± 3.9	31.1 ± 5.9	24.4 ± 5.9	31.9 ± 3.5^{bB}
	F7	0	0	2.2 ± 2.2	0.7 ± 0.7^{eD}

Each experiment data in this table indicators mean ± SEs. Different small and capital letters in the mean column indicate significant and great significant differences at $p < 0.05$ and $p < 0.01$, respectively, based on ANOVA-Duncan multiple comparison results.

External colony morphologies are shown in Fig. 2. The F1 colonies were round with regular edges and vigorous white aerial hyphae (Fig. 2d). The lower surfaces of the colonies were also white (Fig. 2e). The F3 colonies had the same morphology as those of F1 (Fig. 2f) but their lower surfaces had canary yellow patterns (Fig. 2g). The F4 colonies were white and formed rosette patterns (Fig. 2h) and most had white lower surfaces, although a few were canary yellow (Fig. 2i). The F6 colonies were round with white aerial hyphae (Fig. 2j) and gradually developed a deep yellow coloration on their lower surfaces (Fig. 2k). The F2 colonies were round with vigorous lavender aerial hypha (Fig. 2l) and purple lower surfaces (Fig. 2m). The F7 colonies were round (Fig. 2n) with vigorous light pink aerial hyphae (Fig. 2o). The F5 colonies were also round (Fig. 2p) with vigorous fuchsia aerial hyphae, some of which covered with thin white layers. The lower surfaces of the colonies were also fuchsia (Fig. 2q).

Pathogenicity Test Results

Healthy F. przewalskii bulbs were stabbed with cakes of each isolate. Non-stabbed bulbs served as controls (Table 2). Bulbs of all three ages inoculated with the seven strains presented symptoms similar to those observed in the naturally infected F. przewalskii bulbs. Moreover, all strains were isolated from the inoculation sites. A Koch's law analysis indicated that all seven isolated strains were responsible for F. przewalskii bulb rot. By both stabbing and non-stabbing inoculation, F2, F5, and F7 induced the highest bulb rot severity after dark incubation for 4 days. The lesions gradually expanded with incubation time and became bifurcated after 8 days. The incidences of bulb rot induced by F2, F5, and F7 in stabbed bulbs of all ages increased by 75.15%, 76.65%, and 71.44%, respectively, on day 8. Under both the

stabbing and non-stabbing conditions, bulb growth-year had no significant effect on the incidences of these isolates ($F = 0.207$, $P = 0.814$; $F = 0.071$, $P = 0.931$; d.f. = 2,42), however, these incidences were strongly correlated with the isolates themselves ($F = 59.349$, $P < 0.01$; $F = 10.116$, $P < 0.01$; d.f. = 6,42). Significant interactions between growth-year and isolates were not found ($F = 0.446$, $P = 0.934$; $F = 0.162$, $P = 0.999$; d.f. = 12,42) under the two inoculation conditions (Table 2). Bulb rot incidences were significantly higher than those in non-stabbed bulbs of the same age ($t = 16.097$, $P < 0.01$; $t = 15.271$, $P < 0.01$; $t = 12.741$, $P < 0.01$; $n = 9$) (Table 2). The lesions induced by F2, F5, and F7 had completely covered the stabbed bulbs after 10 days of incubation, and 30 days later in the non-stabbed bulbs. However, no lesions were detected in any of the PDA-inoculated controls. The lesions were round or near-circular, moist, and yellow-brown, dark-brown, or blue-black, and their central portions collapsed. The hyphae growing on the non-stabbed bulbs inoculated with F2, F5, and F7 were more vigorous than those on the bulbs infected with the other strains. The F2, F5, and F7 mycelia were thick and mostly white, except for tinges of mauve in F2 and F7 and fuchsia in F5. No lesions were found on the non-stabbed bulbs inoculated with F3, F4, and F6. In contrast, lesions did not appear in the bulbs stabbed with F1, F3, F4, and F6 until the sixth day after inoculation. By the eighth day, the incidences of these isolates increased by 39.09% ($t = 13.456$, $P < 0.01$; $n = 9$), 34.93% ($t = 21.930$, $P < 0.01$; $n = 9$), 40.37% ($t = 16.243$, $P < 0.01$; $n = 9$), and 36.87% ($t = 12.914$, $P < 0.01$; $n = 9$), respectively, compared to the non-stabbed controls (Table 2). All of the lesions produced by F1, F3, F4, and F6 had the same shape but were smaller than those arising from F2, F5, and F7. In the former case, the lesions were moist and yellow-brown but without obvious pitting. They were covered with thin white- to yellow-white hyphae. This morphology indicated very weak pathogenicity.

Table 2

Pathogenic incidence of the strains from different growing years of Fritillaria przewalskii rot bulbs to the 3–5 growing year health ones

Treatments	Incidence rate of the strains from different growing years of rot bulbs (%)			
	3 years	4 years	5 years	Means
Inoculated isolates by bulb stabbing				
F1	40.0 ± 5.1	42.1 ± 4.8	38.9 ± 5.6	40.3 ± 3.2^{bB}
F2	95.0 ± 5.0	86.8 ± 6.8	85.3 ± 7.7	89.2 ± 4.4^{aA}
F3	33.3 ± 0.0	33.3 ± 0.0	38.1 ± 4.8	34.9 ± 2.0^{bB}
F4	40.9 ± 4.9	38.1 ± 4.8	42.1 ± 4.8	40.4 ± 3.0^{bB}
F5	94.8 ± 5.3	90.0 ± 5.1	89.7 ± 10.3	91.5 ± 4.6^{aA}
F6	32.2 ± 1.1	37.2 ± 3.9	41.2 ± 7.8	36.9 ± 3.5^{bB}
F7	90.4 ± 5.0	86.3 ± 3.0	78.2 ± 14.3	85.0 ± 5.8^{aA}
CK(PDA)	0	0	0	$0\ (CK)^{cC}$
Inoculated isolates by bulb non-stabbing				
F1	0	0	3.7 ± 3.7	1.2 ± 1.5^{bB}
F2	15.0 ± 1.7	14.3 ± 7.7	12.8 ± 6.6	14.0 ± 3.6^{aA}
F3	0	0	0	0^{bB}
F4	0	0	0	0^{bB}
F5	11.1 ± 5.6	16.7 ± 0.0	16.7 ± 9.6	14.8 ± 6.1^{aA}
F6	0	0	0	0^{bB}
F7	14.3 ± 2.4	13.9 ± 7.4	12.5 ± 6.4	13.6 ± 3.6^{aA}
CK(PDA)	0	0	0	$0\ (CK)^{bB}$

Each experiment data in this table indicators mean ± SEs. Different small and capital letters in the mean column of bulb stabbing and non-stabbing groups indicate significant and great significant differences at $p < 0.05$ and $p < 0.01$, respectively, based on ANOVA-Duncan multiple comparison results.

Pathogen Identification

The morphologies of the F1, F3, F4, and F6 colonies on PDA medium are shown in Fig. 5. F1, F3, and F4 resembled Bionectria ochroleuca whereas F6 was similar to Clonostachys rosea. These finding spreliminarily confirmed that they were anamorphs (Fig. 2d-2 k). Detailed external colony morphologies of all isolates are shown in Fig. 2 (d-k). Bionectria ochroleuca mycelia was septate and fairly dense. Conidiophores arising from the mycelia generated branching stalks 18.540.0 µm long, 1.52.5 µm wide, and 10.020.0 µm long. The sporogenic cell was 4.07.5 µm long and 1.02.5 µm wide. The conidia were pale, ellipsoid, and 5.06.5 µm × 2.53.0 µm (Fig. 3a-3 h).

The F2 and F7 colonies on PDA medium were round and mostly mauve or slightly pink. Their hyphae were wooly and white or pink to purple. This colony morphology resembles that of Fusarium oxysporum (Fig. 21, 2 m). The lower surfaces of the colonies were dark purple. The microconidia were oval or kidney-shaped and 5.521.5 µm × 2.55.5 µm in size. The macroconidia were sickle-shaped with two to four separations and were 35.045.0 µm × 3.05.0 µm in size. The spherical chlamydospore was interstitial, coniferous, or terminal and had a diameter of 5.58.0 µm (Fig. 3i-3 l).

The F5 colonies on PDA medium were round and mostly burgundy or slightly white and resembled those of Fusarium tricinctum (Fig. 2p, 2q). The lower surfaces of the colonies were burgundy. The hyphae were like cotton wool and burgundy or white. The microconidia were oval or kidney-shaped and either had one septum or none at all. The macroconidiospores were curved or elliptical and had three to five septa. The chlamydospores were spherical and smooth-walled. They were interspersed and either solitary or clustered (Fig. 3m-3p).

Molecular Biology Identification

The genomic DNAs of isolates F1F7 were extracted and used as templates for the PCR amplification of the ITS region; the sequences obtained were 529, 503, 529, 528, 525, 529, and 528 bp (Table 3), respectively, and registered under GenBank Accession Nos. MH917682-MH917688 (Table 4). The BLAST analysis indicated that the DNA sequence of F2 was highly similar to that of Fusarium oxysporum (Accession Nos. JF807396.1 and MG543757.1). The DNA sequence of F7 was highly homologous with that of Fusarium oxysporum (Accession No. JX669526.1). The identity was over 99% in the local alignment (Table 4, Fig. 4) and 96% in the global alignment (Table 4). These clustered into the same group on the phylogenetic tree (Fig. 11) and F2 and F7 were both considered Fusarium oxysporum. The DNA sequence of F5 was 100% homologous to Fusarium tricinctum (Accession Nos. MG547895.1, MF326625.1, MH071359.1, and KP091283.1) with 100% identity in the local alignment (Fig. 4) and over 47% in the global alignment (Table 4). All of these sequences clustered into the same group on the phylogenetic tree and therefore F5 strain was classified as Fusarium tricinctum. The DNA sequences of F1, F3, and F4 were all 100% homologous with Bionectria ochroleuca (Accessions: GU934503.1, AB470910.1, JF311931.1, and FJ426388.1). The F6 sequence was > 99% homologous with that of Clonostachys rosea (Accession No. KJ941018.1) in the local alignment. All of these sequences clustered together in the same group on the phylogenetic tree, confirming that Clonostachys rosea is an anamorph of Bionectria ochroleuca. The colony morphology corroborated the accuracy of the molecular data. The

DNA sequence analysis of the seven strains isolated from F. przewalskii bulb rot lesions revealed that these correspond to the four fungal pathogens known to cause this disease.

TABLE 3 The 18S DNA sequences extracted from ITS region in seven isolates isolated from 3 to 5 growing year rot bulbs of *Fritillaria przewalskii*

Isolate no.	Sequences
F1	CTCCCAACCCATGTGAaCATACCTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTCG TGTGCCCCGGATCAGGCGCCCGCCTAGGAAACTTAATTCTTGTTTTATTTTGGAATCTT CTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCTGA GCGTCATTTCAACCCTCATGCCCCTAGGGCGTGGTGTTGGGGATCGGCCAAAGCCCGC GAGGGACGGCCGGCCCCTAAATCTAGTGGCGGACCCGTCGTGGCCTCCTCTGCGAAGT AGTGATATTCCGCATCGGAGAGCGACGAGCCCCTGCCGTTAAACCCCCAACTTTCCAA GGTTGACCTCAGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAAAACCCCCGG AGGAA
F2	CTCcCAACCCTGTGAaCATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAA ACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAA AACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG AACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT TTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTC AACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCCCAAATTGATT GGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTC GCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATAC CCGCTGAACTTAAGCATATCAAAACCCCCGGAGGAA
F3	CTCCCAACCCATGTGAaCATACCTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTCG TGTGCCCCGGATCAGGCGCCCGCCTAGGAAACTTAATTCTTGTTTTATTTTGGAATCTT CTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCTGA GCGTCATTTCAACCCTCATGCCCCTAGGGCGTGGTGTTGGGGATCGGCCAAAGCCCGC GAGGGACGGCCGGCCCCTAAATCTAGTGGCGGACCCGTCGTGGCCTCCTCTGCGAAGT AGTGATATTCCGCATCGGAGAGCGACGAGCCCCTGCCGTTAAACCCCCAACTTTCCAA GGTTGACCTCAGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAAAACCCCCGG AGGAA
F4	CTCCCAACCCATGTGAACATACCTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTC GTGTGCCCCGGATCAGGCGCCCGCCTAGGAAACTTAATTCTTGTTTTATTTTGGAATCT TCTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCTG AGCGTCATTTCAACCCTCATGCCCCTAGGGCGTGGTGTTGGGGATCGGCCAAAGCCCG CGAGGGACGGCCGGCCCCTAAATCTAGTGGCGGACCCGTCGTGGCCTCCTCTGCGAAG TAGTGATATTCCGCATCGGAGAGCGACGAGCCCCTGCCGTTAAACCCCCAACTTTCCA AGGTTGACCTCAGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAAAACCCCGG AGGAA
F5	TACCTGATcCGAGGTCaACATTCAGAAGTTGGGGTTTTACGGCATGGCCGCGCCGCGTT CCAGTTGCGAGGTGTTAGCTACTACGCAATGGAGGCTGCAGCGAGACCGCCAATGTAT TTCGGGGGCGGCACCGCCCAGAAGGGCAGAGCCGATCCCCAACACCAAACCCGGGGG CTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCGGAATACCAGCGGGCGCA ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT TTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTT ATTTGTTTGTTTTACTCAGAAGTTACAATAAGAAACATTAGAGTTTGGGTCCTCTGGCG GGCCGTCCCGTTTTACGGGGCGCGGGCTGATCCGCCGAGGCAACATTAAGGTATGTTC ACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAGGCCCCCCAACGGAA A
F6	CTCCCAaACCCATGTGAaCATACCTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTC GTGTGCCCCGGATCAgGcGCCCGCCTAGGAAACTTAATTCTTGTTTTATTTTGGAATCTT CTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCACTGAATC ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCTGA GCGTCATTTCAACCCTCATGCCCCTAGGGCGTGGTGTTGGGGATCGGCCAAAGCCCGC GAGGGACGGCCGGCCCCTAAATCTAGTGGCGGACCCGTCGTGGCCTCCTCTGCGAAGT AGTGATATTCCGCATCGGAGAGCGACGAGCCCCTGCCGTTAAACCCCCAACTTTCCAA GGTTGACCTCAGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAAAACCCCGGA GGAA
F7	CTCcCAACCCTGTGAaCATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAA ACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAA AACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG AACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT TTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTC AACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATT GGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTC GCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATAC CCGCTGAACTTAAGCATATCAAAACCCCGGAGGAA

Table 4

Pairwise nucleotide sequence alignment identity (%) among 33 accessions including species Bionectria ochroleuca (Boc), Clonostachys rosea (Cro), Fusarium sp.(F), Fusarium avenaceum (Fav), Fusarium acuminatum (Fac), Fusarium oxysporum (Fox), Fusarium tricinctum (Ftr) retrieved from GenBank compared to seven strains (F1, F2, F3, F4, F5, F6, F7) originally isolated from Fritillaria przewalskii Maxim. rot bulbs with Needleman-Wunsch Global Alignment in NCBI

Accession (genotype)	Species	SEq. length	F1	F2	F3	F4	F5	F6	F7
MH917682 (F1)	Boc	529	100	78.8	100	99.8	46.9	99.4	78.4
MH917683 (F2)	Fox	502	78.8	100	78.8	78.6	48.7	78.0	99.6
MH917684 (F3)	Boc	529	100	78.8	100	99.8	46.9	99.4	78.4
MH917685 (F4)	Boc	528	99.8	78.6	99.8	100	46.7	99.6	78.5
MH917686 (F5)	Ftr	525	46.9	48.7	46.9	46.7	100	46.7	48.9
MH917687 (F6)	Cro	529	99.4	78.0	99.4	99.6	46.7	100	78.0
MH917688 (F7)	Fox	502	78.4	99.6	78.4	78.5	48.9	78.0	100
AB470910.1 (X1)	Boc	528	98.9	77.7	98.9	99.1	46.8	98.7	77.6
FJ426388.1 (DB-5B)	Boc	540	96.7	76.0	96.7	96.9	46.3	96.5	75.9
GU934503.1 (OTU430)	Boc	584	89.7	70.4	89.7	89.9	46.1	89.9	70.4
JF311931.1 (P295_D3_10)	Boc	538	91.9	71.7	91.9	92.1	45.5	91.7	79.8
JF776663.1 (WAC:11486)	Ftr	561	74.3	79.9	74.3	74.4	48.5	74.4	79.8
JF807396.1 (K9)	Fox	517	76.1	96.3	76.1	76.2	48.8	75.9	96.3
JQ301898.1 (BWH-Z)	Fox	523	75.0	95.0	75.0	75.2	49.0	74.9	95.4
JQ690084.1 (ZD)	Ftr	565	74.5	80.1	74.5	74.4	48.2	74.4	79.7
JX669525.1 (SY-1)	Fox	521	75.3	95.4	75.3	75.5	49.1	75.1	95.8
JX669526.1 (SY-2)	Fox	519	75.6	95.8	75.6	75.7	49.1	75.7	96.2
KC767895.1 (cjl40804)	Ftr	547	74.0	79.6	74.0	74.2	48.2	74.2	79.6
KF939132.1 (1)	Fav	553	76.0	81.7	76.0	76.1	48.8	76.1	81.6

Numbers underlined in each column indicate the highest and lowest identities in each bulb rot strains.

Accession (genotype)	Species	SEq. length	F1	F2	F3	F4	F5	F6	F7
KJ941018.1 (NEFU)	Cro	559	93.8	73.5	93.8	93.9	45.8	93.9	73.5
KP091283.1 (LZF1)	F	538	73.9	79.5	73.9	74.0	47.6	73.9	79.5
KR817677.1 (6-5-1)	Ftr	542	74.1	79.7	74.1	74.2	48.0	74.2	79.7
KU892283.1 (2015-F-366)	Ftr	545	74.3	79.9	74.3	74.4	47.8	74.4	79.9
KX058060.1 (fsp15)	Fox	531	72.6	78.2	72.6	72.7	47.8	72.7	78.2
KY078213.1 (ZJU-1)	Fox	520	75.5	95.6	75.5	75.6	49.3	75.3	96.0
KY365570.1 (D22-2)	Fac	545	74.7	80.4	74.7	74.9	48.0	74.9	80.4
LC383470.1 (BRSP02)	Fox	571	69.3	87.4	69.3	69.3	48.8	69.3	87.6
MF523230.1 (LD1508081502)	Fac	544	74.7	80.4	74.7	74.9	48.2	74.9	80.4
MF326625.1 (IHBF 2204)	Ftr	542	74.3	79.9	74.3	74.4	48.2	74.4	79.9
MF457482.1 (KB3)	Fox	557	70.6	89.1	70.6	70.7	48.3	70.7	89.1
MF567508.1 (dmSON)	Cro	613	85.5	67.1	85.5	85.6	45.7	85.6	67.0
MF663693.1 (VGCR16-3)	Cro	525	98.7	77.2	98.7	98.9	46.9	98.5	77.2
MF671812.1 (VGCR15-7)	Cro	527	98.9	77.4	98.9	99.1	46.7	98.7	77.4
MF671813.1 (VGCR15-10)	Cro	527	98.9	77.4	98.9	99.0	46.7	98.7	77.4
MG543757.1 (LrBF39)	Fox	519	76.0	96.2	76.0	76.1	49.2	75.9	96.2
MG547895.1 (no)	Ftr	569	73.6	79.1	73.6	76.1	49.2	73.8	79.0
MH071359.1 (PFS1)	Ftr	550	73.6	79.2	73.6	73.8	47.9	73.8	79.2
MH071363.1 (PFS6)	Ftr	666	63.7	68.3	63.7	63.6	45.6	63.6	54.4
MH424135.1 (CR-BRK1)	Cro	758	69.4	54.7	69.4	69.3	42.3	69.2	54.4
MH424143.1 (CZ-44)	Cro	719	73.2	57.7	73.2	73.2	43.3	73.0	57.5

Numbers underlined in each column indicate the highest and lowest identities in each bulb rot strains.

Discussion

Bulb rot pathogens accumulate differently with F. przewalskii growth

Bulb or root rot are soil-borne diseases and major causes of the yield and quality loss of the liliaceous onion and shallot [24], garlic [25], also loss for rhizomes medicinal plants [8, 9]. Although surviving in soil for a long time, the species and quantity of rot pathogens are affected by climate, succession of soil types, and biological factors [9, 26]. The dominant pathogens causing rot may differ among regions or years [27]. Rojas et al. [28] found that rhizosphere and bulk soils harbored distinct microbial communities within the same region, and this is why we observed diverse bulb rot pathogens in our wild fostered fields of the rare medicinal plant F. przewalskii. Inter-annual differences in the fungal communities might also have produced diverse levels of individual disease resistance, resulting in only a few bulbs being infected within the same year, and then gradually expanding: only four pathogenic strains (F1, F2, F5, and F6) were detected in the3-year-old bulbs but this number increased to six in 4-year-old bulbs, with the detection of F3 and F4, and to seven in 5-year-old bulbs with the detection of F7. These pathogens reproduced to a certain extent leading to bulb rot. However, no disease was detected in 1 or 2-year-old bulbs suggesting that infection risk increases with bulb age. Once the bulb is injured, the wounds attract pathogens from the soil causing bulb rot, especially when the soil microclimateis conducive to pathogen development. Although all detected strain caused F. przewalskii bulb rot, their pathogenicity varied: F5 showed the strongest pathogenicity, followed by F2 and F7, and then by F1, F3, F4, and F6. The initial pathogenicity significantly differed among strains, which may be the result of micro environmental differences, as the bulb rot was promoted during summer in drought and low-lying rocky soils

Strains polymorphism indicates their ability infect hosts in different growth-years

Plant pathogenic fungi were traditionally classified and identified according to their vegetative organs and fruiting bodies [8]. However, this method is not always reliable because morphological characteristics are not necessarily consistent for the same fungal species. In contrast, molecular methods may identify fungal species rapidly and accurately [29]. In addition, eukaryotic DNA base composition is genetically stable, resistant to environmental influences, and can be determined at any growth stage [30]. The eukaryotic rDNA-ITS region is a non-coding region with a relatively conserved sequence. However, due to different degrees of mutation at the inter- and intraspecies levels, ITS sequencing may be effective for classifying and identifying species [30]. In our study, we further identified the strains based their ITS sequences and on phylogenetic analysis, all coincident with the morphological identification. The sequences obtained for the seven pathogenic strains were deposited in GenBank under accessions MH917682-MH917688, and were identified as Bionectria ochroleuca (F1, F3, F4), Fusarium oxysporum (F2, F7), Fusarium tricinctum (F5), and Clonostachys rosea (F6). Polymorphic strains for one pathogenic species indicates their ability to infect hosts at different growth-years to ensure survival. Strain F1 of Bionectria ochroleuca appeared in the 3-year-old bulbs while strains F3 and F4 were only detected in 4-

and 5-year-old bulbs. Similarly, strain F7 of Fusarium oxysporum appeared only in 5-year-old bulbs, while its homologous strain F2 was found in bulbs of all three growing-years.

Identified pathogens cause rare medicinal F. przewalskii bulb rot but Fusarium sp. are dominant

Fusarium sp. are the most common pathogens causing root or bulb rot, as their mycelia and chlamydial spores can survive for many years in the soil and can destroy their hosts' vascular tissues. Fusarium sp. have been isolated from the diseased organs of many crops [31–34] where they cause only a moderate degree of rot, ranging from 0.98–30.39% [32]. In our study, three strains (F2, F5, F7) identified as Fusarium sp. were recovered from diseased bulb samples collected from 3- to 5-year-old F. Przewalskii (Chuanbeimu).Two species of Fusarium were identified, with Fusarium tricinctum (F5) being predominant (76.65%), followed by Fusarium oxysporum strains F2 and F7 (75.15% and 71.44%, respectively). This could be attributed to the different aggressiveness of species isolates [36, 37]. Fusarium oxysporum also causes bulb rot of F. hupehensis Hsiao et K.C.Hsi (Hubeibeimu) [18]. In addition to being infected by Fusarium tricinctum, and differing from Hubeibeimu in this respect, Chuanbeimu commonly harbors two morphologically different strains of Fusarium oxysporum (F2, F7) as well as other two fungal species of different genera. Additionally, our results differed from that of Supi et al. [16] who reported that Fusarium solani var coeruleum caused F. pallidiflora Schrenk (Yibeimu) bulb rot in Xinjiang Province of China. Only two related sequences of this pathogen were deposited in GenBank (Accessions: KP017789.1 and KP017788.1), and their highest similarity with F1-F7 strains in our study was 71%. This also confirmed that the pathogens causing bulb rot vary with region and species [26].

Fusarium oxysporumis one of the most economically important fungus within the genus [37] and it forms a heterogeneous species complex composed of numerous species that need to be identified. Plant pathogenic representatives of the Fusarium oxysporum species complex are mostly associated with vascular wilt [38], damping-off [8], and crown and root rots [39, 40]. Furthermore, many isolates within this species complex are host specific [9, 12, 13, 15]. Therefore, this species complex has wide host range and it is relatively stable in the environment. In our study, we also found that two strains of Fusarium oxysporum were highly pathogenic to F. przewalskii bulbs (85.0% and 89.2%) in agreement with results of previous studies.

Clonostachys rosea and Bionectria ochroleucaare necrotrophic mycoparasitic fungi used for biological control of other plant pathogenic fungi in agriculture and horticulture [41–44]. Preliminary experiments with carrot also found that Clonostachys rosea biopriming reduced seedling damping-off caused by Pythium ultimum mixed in the growing media (B. Jensen, unpublished data). In field experiments, spraying with Clonostachys rosea conidia reduced leaf and head rot of Chinese cabbage caused by Pythium tracheiphilum [45]. However, in our study, Clonostachys rosea was mildly pathogenic to F. przewalskii, mainly in vading it through wounds. In fact, F. przewalskii bulb rot seems to result from seven pathogenic strains, which infect this host in different growth-years. Thus, when fostered in fields, the bulbs must be operated gently to avoid wounds.

Overall, our study showed that the seven fungal strains isolated from the rot bulbs of 3 to 5-year-old rare medicinal F. przewalskii plants belong to four species, which were not detected in 1 to 2-year-old plants. As the rhizospheric microenvironment changes, and the host plant affects this alteration, there are differences in the fungal strains causing bulb rot. Therefore, pathogenic fungi might not be easy to distinguish based solely on plant age. Additionally, the differences among fungal isolates found in 3- to 5-year-old bulbs suggest the occurrence of bulb rot patterns for this rare and endangered medicinal plant, providing basis for its conservation in fostered fields. So far, this is the first report on F. przewalskii bulb rot in China. Further work is needed to investigate the precise pathogenic mechanisms responsible for bulb rot induction in this alpine plant under pathogen mixed-infection conditions. The present results may help developing the breeding and cultivation of F. przewalskii protecting it against bulb rot and provide an explanation for the extinction of this rare medicinal plant.

Conclusions

A total of 7 fungus strains were isolated from diseased plant samples with bulb rot symptom of the 3 to 5-year growing plants of F. przewalskii by tissue separation. Phylogenetic analyses, in conjunction with morphological characters, revealed that theses fungus strains were Bionectria ochroleuca, Fusarium oxysporum, Fusarium tricinctum and Clonostachys rosea. Pathogenicity tests found that Fusarium tricinctum and Fusarium oxysporum had the strongest pathogenicity to the health bulb, followed by Bionectria ochroleuca and Clonostachys rosea. Although leading to low bulb rot incidence by stab-inoculation, Bionectria ochroleuca showed the highest isolation rate among all strains. Therefore, when fostering in field, the bulbs must be operated gently to avoid to wounds.

Abbreviations

F.: Fritillaria; SE: Stand Error; PDA: potato dextrose agar; ISF: isolate separation frequency; In: the number of isolates with the same morphology; TB: the total bulb blocks in each culture dish for each growing-year bulb group; WA: water agar; ITS: internal transcribed spacer; PCR: Polymerase Chain Reaction; BLAST: Basic Local Alignment Search Tool; BLAST: Basic Local Alignment Search Tool; DNA: Deoxyribonucleic acid.

Declarations

Acknowledgements

The authors thank Prof. Shengrong Wang for help in strains identification, also thank Dr. Anim Samuel, and Master Zhou Yang, Wang Hongyan and Bachelor student Jingcheng Zhou for their diligent field work. The authors still expressed thanks to Junyan Kang and Zhijun Guo for their skillful technical assistance in field cultivation and management.

Authors' contributions

YC collected and provided the plant seeds, designed, coordinated, and supervised the study. RW, FXG and YG performed the experiments. FXG and RW analyzed the data and drafted the manuscript. FXG and LLD made revisions. All authors read and approved the manuscript.

Funding

This study was supported by the National Natural Science Foundation of China (31660158 and 31360316), Gansu Key Science & Technology Program of Chinese Medical Industry (GYC12-06) and Chief Expert of Traditional Chinese Medicinal Industry in Modern Agricultural Industry System (GARS-ZYC-1) provided by Gansu Provincial Department of Agriculture and Pastoral Affairs as.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China (one). Beijing: China Medical Science Press. 2015.
2. Wang S, Xu XP, Li T. Determination and contrastion of alkaloids and saponins in bulbus fritillariae cirrhosae and in other beimus. China Journal of Chinese Materia Medica. 2002; 27(5):342-344.
3. Liu J, Peng C, He CJ, Liu JL, He Y.C, Guo L,et al. New amino butenolides from the bulbs of *Fritillaria unibracteata*. *Fitoterapia*. 2014;98:53-58.
4. Chang YL, Chen Y, Guo FX, Lin YH, Li T. A study on soaking and germination characteristics of *Fritillaria przewalskii* seeds. Acta Prataculturae Sinica. 2010;19(4):41-46.
5. Chen Y, Xu BQ, Guo, FX. Bai G, Zhang JF, Zhang Y. Studies of the floral organ characteristics and sexual breeding system of *Fritillaria unibracteata*. Acta Prataculturae Sinica. 2017;26(1):90-98.
6. Guo FX, Chang, Y.L, Lin Y.H, Wang S X, Zhu J, Chen Y.. Studies on grain filling characteristics of *Fritillaria przewalskii*. Acta Prataculturae Sinica. 2010;19(2):97-102.

7. Arfaoui A, Adam, L. R., Bezzahou, A., & Daayf. F. Isolation and identification of cultivated bacteria associated with soybeans and their biocontrol activity against *Phytophthora sojae*. Biocontrol. 2018;63(7):607-17.
8. Liu YY, Chen Y, Guo FX, Shi YT, Li ZX, Li HM. Isolation and identification of the pathogens causing *Rheum palmatum* root rot. Acta Prataculturae Sinica. 2011;20(1):199-205.
9. Chen Y, Zhu L, Guo FX, Wu Z J. Isolation and identification of the pathogens causing *Astragalus membranaceus* var. mongholicus root rot in Weiyuan of Gansu Provinc*e. Acta Phytopathologica Sinica.* 2011;41(4):428-431.
10. Lu ZK, Wang GZ, Zhang LR, Liang J, Ru QH. Occurrence and control of wolfberry root rot. Journal of Plant Protection. 1994;21(3):249-254.
11. Li H, Li GY, Fu JH. Identification of the causing agent of wolfberry root rot in Xinjiang. Journal of Plant Protection. 1998;25(3):253-257.
12. Li J, Feng LD, Wang YK. He J, Chen X.R. Identification and biological characteristics of dominant pathogens of *Lycium bararum* root rot in Gansu Province. Arid Zone Research. 2017;34(5):1093-1100.
13. Niu SQ, Geng H, Han CH, Yan WR, Da WY. Isolation and identification of pathogens causing *Astragalus membranaceus* root rot in Gansu Longxi. Journal of Northwest Normal University. 2016;52(2):75-78.
14. Ren XX, Qin XM, Wang ML, Lei ZH, Wang YL, Gao F. Identification of dominant pathogen causing root rot disease in *Astragalus membranaceus* var. mongholicus of Shanxi Province and screening of antagonistic microorganism. Journal of Chinese Medicinal Materials. 2016;39(10):2173-2175.
15. Tang XM, Lin TY, Zhou SS, Li GF, Liu P, Ye ZF, et al. Pathogen identification of root rot in pear plant and fungicide screening for its efficient control. Journal of Nanjing Agricultural University. 2017;40(1):76-83.
16. Supi R, Sulaiman K, Aybiek. Research on identification of pathogeny and biological characteristics of bulbusFritillaria root rot. Tianjin Agricultural Sciences. 2015;21(3): 118-121.
17. Zhou MF, Lu HJ, Yang D, Li X, Zhao CS. Studies on the cause of bulb rot of*Fritillaria hypehensis*and its integrated control. Journal of Huazhong Agricultural.1989;8(1):23-28.
18. Booth C. The genus fusarium Commonwealth Mycological Institute. England: Kew Surrey. 1971.
19. Fang ZD. Methods for research on plant pathology. Beijing: China agricultural press.1998;122-125.
20. Zhong S, Zhang T, Yang J, Sun H, Zhang GZ. Identification of pathogen causing straw berry Rhizoctonia root rot. Acta Phytopathologica Sinica. 2016;46(3):289-293.
21. Xie HT, Tan HW, Tang XL, Chen DM, Li QQ, Yuan GQ, Lin W. Identification of root rot pathogen of *Rosmarinus officinalis* L.. Acta Phytopathologica Sinica. 2013;43(2):211-214.
22. Dewhirst FE, Shen Z, Scimeca MS, Stokes LN, Boumenna T, Chen T, et al. Discordant 16S and 23S rRNA gene phylogenies for the genus Helicobacter: implications for phylogenetic inference and systematics. *Journal of Bacteriology*. 2005;187(17):6106-6118.

23. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution. 2011;28(10):2731-2739.
24. Sintayehu A, Sakhuja PK, Fininsa C, Ahmed S. Management of *Fusarium* basal rot (*Fusarium oxysporum* f. sp. cepae) on shallot through fungicidal bulb treatment. Crop Protection. 2011;30(5):560-565.
25. Gálvez L, Urbaniak M, WaśKiewicz A, Stępień Ł, Palmero D. *Fusarium proliferatum* causal agent of garlic bulb rot in spain: genetic variability and mycotoxin production. Food Microbiology. 2017;67:41-48.
26. Mandeel QA. Biodiversity of the genus Fusarium in saline soil habitats. Journal of Basic Microbiology. 2006;46(6):480-494.
27. Cheng L, Fang C W, Chen N, Wang J, Ye S.M, Wu JH. Identification of root rot pathogen *Paeonia ostii* T.Hong et J.X.Zhang in Bozhou. Chinese Agricultural Science Bulletin. 2015; 31(16): 190-193.
28. Rojas X, Guo J, Leff JW, Mcnear DH, Fierer N, Mcculley RL. Infection with a shoot-specific fungal endophyte (epichloë) alters tall fescue soil microbial communities. Microbial Ecology. 2016;72(1):197-206.
29. Ramchandra SS, Bhatt P.N. Morphological and molecular identification ofFusarium isolated from cumin wilt. International Journal of Plant Protection. 2011;4(2):359-362.
30. Li LY, Xiao HJ, Wang WQ, Pan Y, Kong QS. Isolation and molecular identification of pathogenic bacteria of sweet potato root rot disease. Current Biotechnology. 2016;6(1): 67-70.
31. Nirenberg HI, Schmitzelsherif H, Kling CI. Occurrence of fusaria and some "blackening moulds" on durum wheat in Germany. I. Incidence of Fusarium species. Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz. 1994;101(5):449-459.
32. Zhou Q, Li NN, Chang KF, Hwang SF, Strelkov SE, Conner RL, et al. Genetic diversity and aggressiveness of, *Fusarium*, species isolated from soybean in alberta, Canada. Crop Protection. 2018;105:49-58.
33. Carrieri R, Raimo F, Pentangelo A, Lahoz E. Fusarium proliferatum and *Fusarium tricinctum* as causal agents of pink rot of onion bulbs and the effect of soil solarization combined with compost amendment in controlling their infections in field. Crop Protection. 2013;43(43):31-37.
34. Castañares E, Stenglein SA, Dinolfo MI, Moreno MV. *Fusarium tricinctum* associated with head blight on wheat in Argentina. Plant Disease. 2011;*95*:496.
35. Skovgaard K, Bødker L, Rosendahl S. Population structure and pathogenicity of members of the Fusarium oxysporum complex isolated from soil and root necrosis of pea (*Pisum sativum* L.). FEMS Microbiology Ecology. 2002;42:367-374.
36. Chittem K, Mathew FM, Gregoire M, Lamppa RS, Chang YW, Markell S.G, et al. Identification and characterization of *Fusarium* spp. associated with root rots of field pea in North Dakota. European Journal of Plant Pathology. 2015;143(4):641-649.

37. Leire, MR, Encarnación RP, Elisa GD, Basallote UMJ. Alternative hosts for *Fusarium* spp. causing crown and root rot of Asparagus in Spain. Journal of Phytopathology. 2011;159(2): 114-116.
38. Huang LW, Wang CJ, Lin YS, Chung WC, Chung WH. Stem rot of jewel orchids caused by a new forma specialis, *Fusarium oxysporum* f. sp. anoectochili in Taiwan. Plant Pathology. 2014;63(3):539-547.
39. Edel-Hermann V, Gautheron N, Steinberg C. Genetic diversity of *Fusarium oxysporum* and related species pathogenic on tomato in Algeria and other Mediterranean countries. Plant Pathology. 2012;61(4):787-800.
40. Leire MR, Encarnación RP, Elisa GD, Basallote UMJ. Alternative hosts for *Fusarium* spp. causing crown and root rot of Asparagus in Spain. Journal of Phytopathology. 2011; 159(2):114-116.
41. Lahlali R, Peng G. Suppression of clubroot by*Clonostachys rosea*via antibiosis and induced host resistance. Plant Pathology. 2014;63(2):447-455.
42. Maria A, Domenica A, Stefania B,Giulia B, Alba B, Daniela B, et al. Peer review of the pesticide risk assessment of the active substance Clonostachys rosea strain J1446. European Food Safety Authority Journal. 2017;15(7):4905.
43. Nygren K, Dubey M, Zapparata A, Iqbal M, Tzelepis GD, Durling MB, et al. The mycoparasitic fungus C*lonostachys rosea* responds with both common and specific gene expression during interspecific interactions with fungal prey. Evolutionary Applications. 2018;11:931-949.
44. Jensen B, Knudsen IMB, Madsen M, Jensen DF. Biopriming of infected carrot seed with an antagonist, *Clonostachys rosea*, selected for control of seedborne *Alternari*a spp. Phytopathology. 2004;94(6):551-560.
45. Møller K, Jensen B, Andersen HP, Stryhn H, Hockenhull J. Biocontrol of *Pythium tracheiphilum* in Chinese cabbage by *Clonostachysrosea* under field conditions. Biocontrol Science and Technology. 2003;13:171-182.

Figures

Fig. 1 (a, b, c, d, e, f, g, h, I, j, k , l)

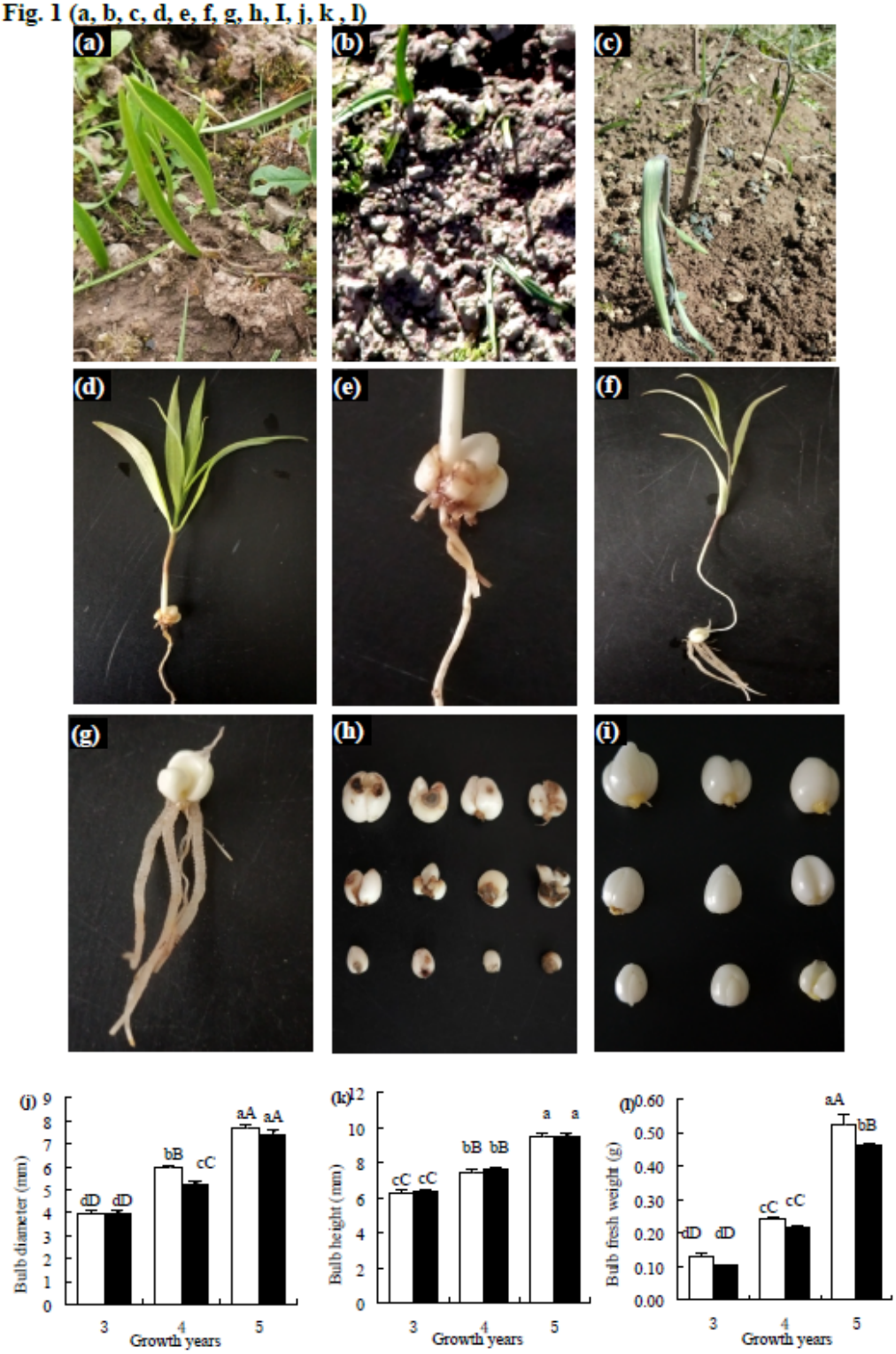

Figure 1

Aboveground phenotype of Fritillaria przewalskii plants artificially fostered in a high-altitude area (2,927 m) in Zhang County, Gansu Province, China. (a) Three-year-old healthy and wilting plants. (b) Four-year-old healthy and wilting plants. (c) Five-year-old healthy and wilting plants. (d) Plant with bulb rot disease. (e) Decayed bulb and roots. (f) Plant with healthy bulbs. (g) Healthy bulb and roots. (h) Rot bulbs with lesions. (i) Healthy bulbs without lesions. (j), (k), and (l) Comparison of bulb diameter, height, and fresh

weight in 3- to 5-year-old bulbs from healthy (□) and rot (■) plants. Different small and capital letters on the six columns indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, based on Duncan's multiple comparisons after analysis of variance. [Color images can be viewed at wileyonlinelibrary.com]

Fig. 2 (a, b, c, d, e, f, g, h, i, j, k)

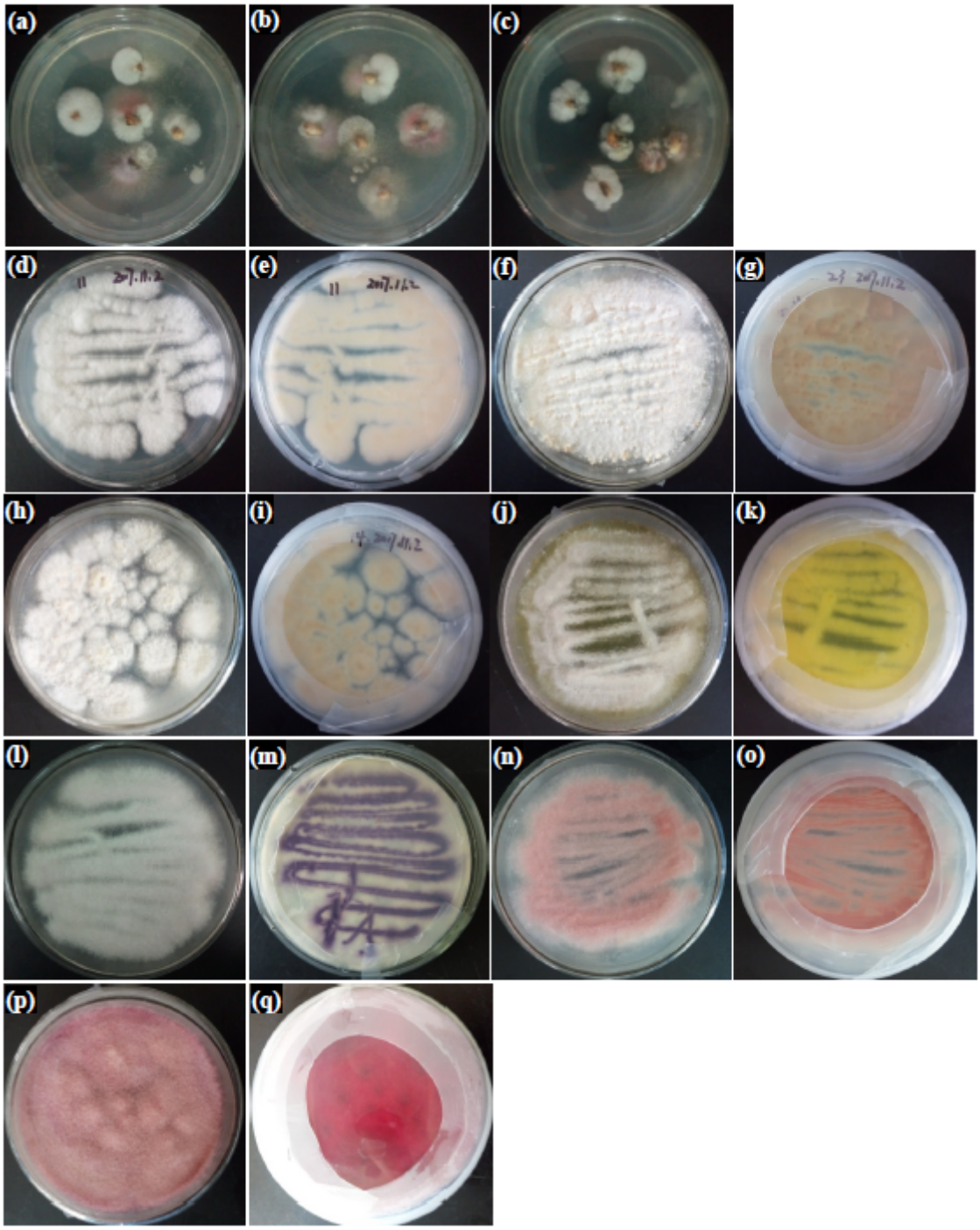

Figure 2

Fungal strains initially isolated from the diseased bulbs of 3- to 5-year-old Fritillaria przewalskii artificially fostered in China. Pathogens were isolated from bulb lesions and incubated in PDA medium in the dark for 4 days. (a) All strains (F1-F7) were present in the diseased 5-year-old bulbs. (b) F1-F6 was found in the

diseased 4-year-old bulbs. (c) F1, F2, F5, and F6 occurred in the diseased 3-year-old bulbs. (d) Top view of F1 colony characteristics on the culture plate. (e) Bottom view of F1colony characteristics on the culture plate. (f) Top view of F3 colony characteristics on the culture plate. (g) Bottom view of F3 colony characteristics on the culture plate. (h) Top view of F4 colony characteristics on the culture plate. (i) Bottom view of F4 colony characteristics on the culture plate. (j) Top view of F6 colony characteristics on the culture plate. (k) Bottom view of F6 colony characteristics on the culture plate. (l) Top view of F2 colony characteristics on the culture plate. (m) Bottom view of F2 colony characteristics on the culture plate. (n) Top view of F7 colony characteristics on the culture plate. (o) Bottom view of F7 colony characteristics on the culture plate. (p) Top view of F5 colony characteristics on the culture plate. (q) Bottom view of F5 colony characteristics on the culture plate. [Color images can be viewed at wileyonlinelibrary.com.]

Fig. 3 (a, b, c, d, e, f, g, h, I, j, k, l, m, n, o, p)

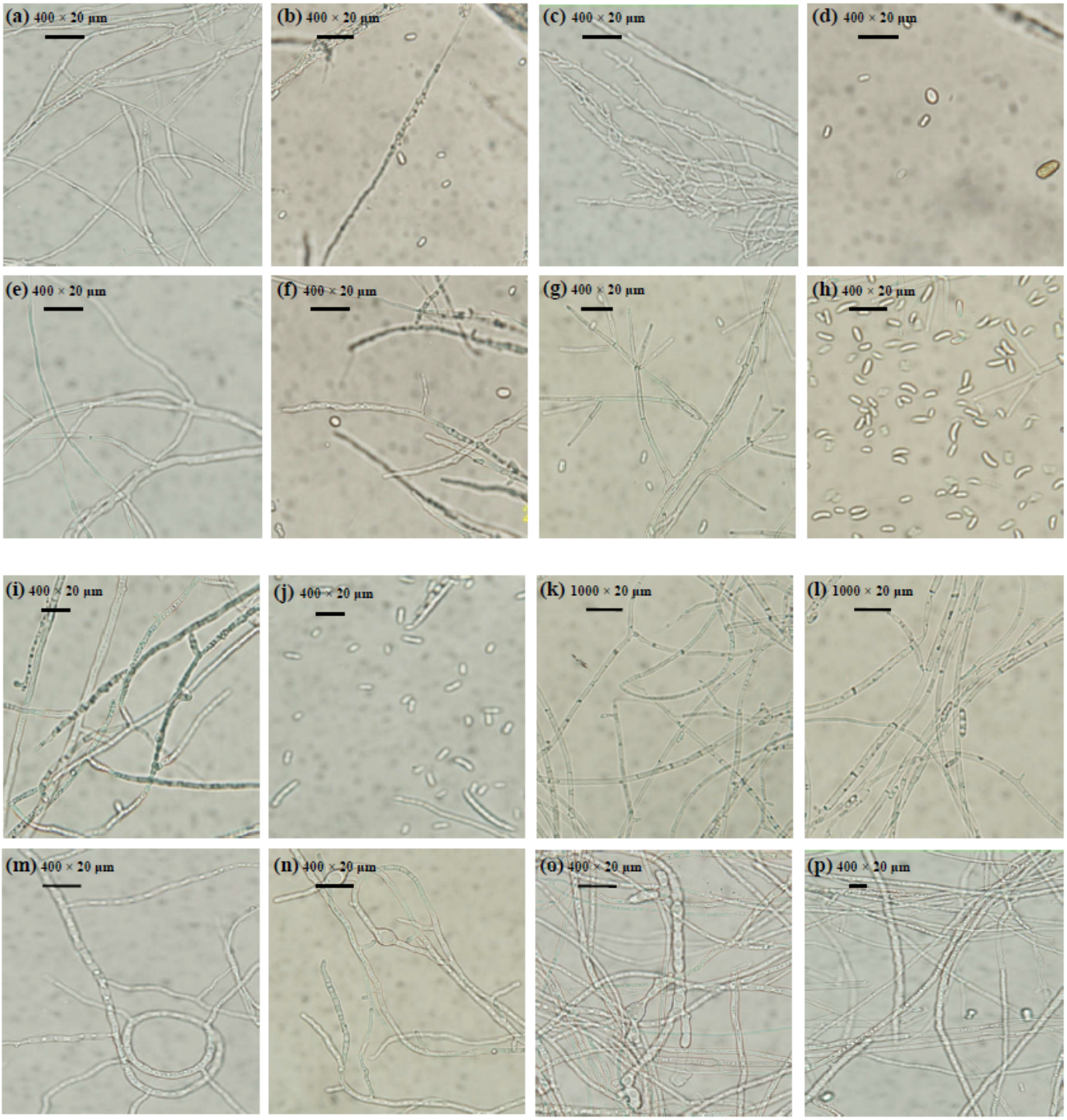

Figure 3

Microscopic morphology of Clonostachys rosea strains F1, F3, and F4 and Bionectria ochroleuca strain F6isolated from diseased Fritillaria przewalskii bulbs and grown on PDA culture plates. (a) Mycelium of F1. (b) Macrospore and microspore of F1. (c) Mycelium of F3. (d) Macrospore and microspore of F3. (e) Mycelium of F4. (f) Macrospore and microspore of F4. (g) Mycelium of F6. (h) Macrospore and microspore of F6. (i) Mycelium of F2. (j) Macrospore and microspore of F2. (k) Mycelium of F7. (l)

Macrospore and microspore of F7. (m) Mycelium of F5. (n) Chlamydospore of F5. (o) Conidiophores of F5. (p) Macrospore and microspore of F5. Photographs were taken at 400×. Scale bars = 20 μm.

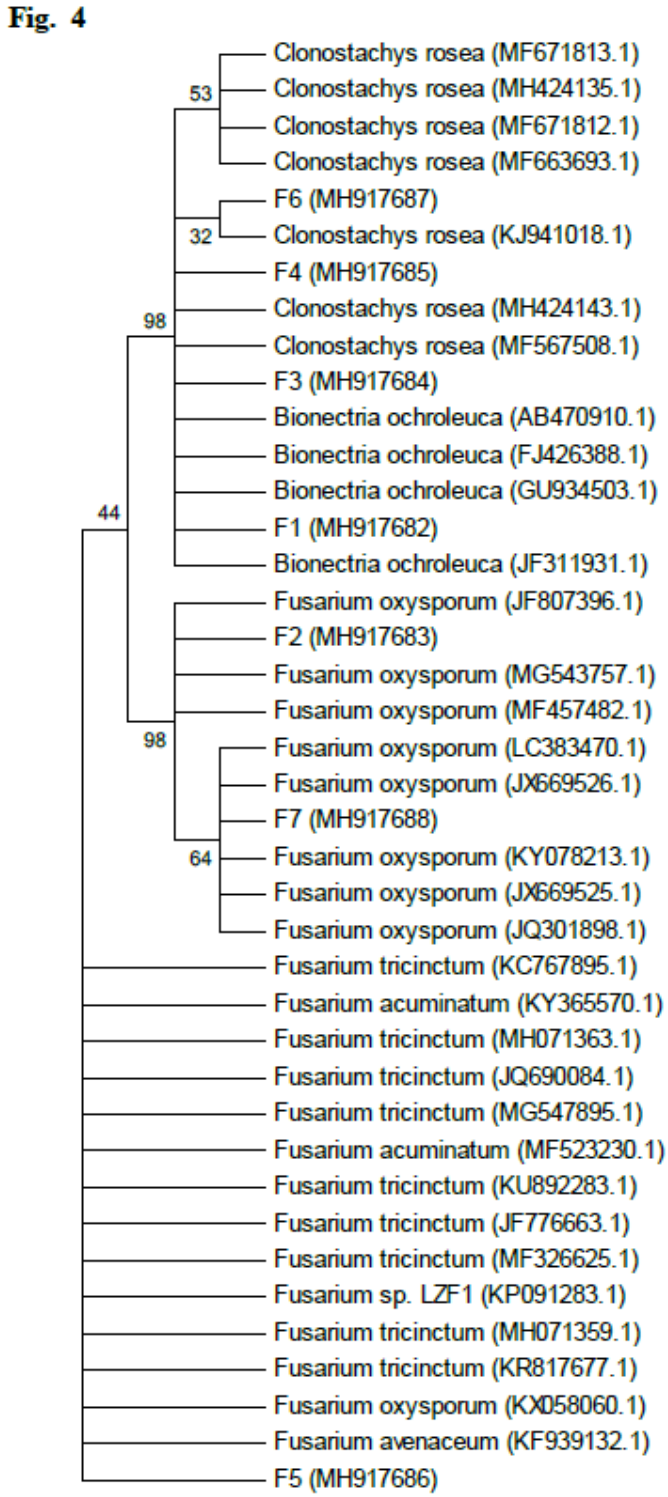

Figure 4

Phylogenetic tree of F1 F7 (originally isolated from diseased Fritillaria przewalskii bulbs) and the species to which they are closely related according to rDNA-ITS sequences.