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A new record of *Fusarium metavorans* (Nectriaceae, Hypocreales) frequent opportunist fungus from Kashmir Himalaya, India

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Abstract

In the course of the mycological survey in several Kashmir Himalayan regions, the collection and isolation of various soil fungi from various sites were performed. The rhizosphere of the important medicinal plant *Digitalis purpurea* L. from the Kashmir Himalaya was used for the first time to isolate *Fusarium metavorans*. To isolate the fungi that were present in the soil samples, the serial dilution method was used. Identification of the fungi was carried out based on cultural, microscopic, and molecular characteristics using the ITS gene. Colonies were white and cottony on the upper side and light yellow to yellowish orange on the reverse side. Aerial mycelium, macroconidia as well as microconidia were present. Established upon the closest identification of BLAST analysis, the nucleotide sequences of 20 isolates obtained from GenBank were compared to the GenBank databases and matched at the diverse global similarity. A literature survey was carried out, and we concluded that *Fusarium metavorans* isolated in our study was a new record from the Kashmir Himalaya, India.

Keyword – BLAST – *Digitalis purpurea* – GenBank – Rhizosphere

Introduction:

Fusarium is one of the best-known and widely dispersed genera in the Fungi kingdom. *Fusarium* includes a vast number of morphologically and phylogenetically diverse species (Leslie & Summerell 2008, Aoki et al. 2014). Although some *Fusarium* species are saprophytes, many are well-known for causing plant infections that can also act as secondary invaders and are significant producers of mycotoxins that can contaminate food, particularly stored seeds (Bhat et al. 2010). Wilting, root and crown rot are diseases caused by *Fusarium* spp. incarnations that inflict damage on greenhouses every year. In most carnation-growing locations of the world, *fusarium* wilt is one of the most devastating diseases (Kermajany et al. 2017). The shape, growth of chlamydospores, colony characteristics (form, color, and presence of diffusible pigments), the presence or absence of sporodochia, and the kind of macro-and microconidia are all useful morphological features (Leslie & Summerell 2006). DNA-based methods, such as conserved sequencing areas, can be used to make molecular identification (Geiser et al. 2004).

Materials and Methods

Collection of Soil Samples

The *Fusarium* strain was isolated from various soil samples taken from the rhizospheric area

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of the medicinal plant, *Digitalis purpurea* L. at various locations around the Kashmir valley. The isolated fungus was identified based on morpho-anatomical and molecular data.

Isolation of the fungi associated with the rhizosphere of *Digitalis purpurea* L. was done by dilution method (Waksman 1994, Warcup 1950). In this method, (1g) of soil was taken from the soil sample. This 1g of soil sample was dissolved in 10 ml of distilled water in sterilized test tubes to get 10^{-1} dilution. From this, 1ml of soil suspension was taken and added to 9 ml of distilled water to get 10^{-2} dilution. The process was repeated until a final dilution of 10^{-6} was obtained. Dilutions of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were used to isolate fungi to avoid the over-crowding of fungal colonies. One milliliter of soil suspension from each concentration was inoculated into new petri plate. Plates were rotated gently to get uniform distribution of soil suspension into the medium. Streptomycin solution (1%) was added to the medium for preventing bacterial growth, before pouring it into petri plates. Then the plates were incubated at 25±2ºC for 5–7 days. These petri plates were then observed for fungal growth and the numbers of fungal colonies were recorded, especially at higher dilutions. Inoculation was done in a laminar airflow chamber on 10 ml of Potato Dextrose Agar medium or Richard's media in replicates for each dilution. Living culture was deposited at fungal section of Kashmir University Herbarium (KASH), Srinagar, India under accession number HNY. KASH-2639.

Molecular identification

DNA isolation and PCR amplification

The usual phenol/chloroform extraction procedure was used to extract genomic DNA from a 15-day-old culture (Sambrook et al. 1989), followed by PCR amplification of the ITS region using the primers ITS1F [5'-TCC GTA GGT GAA CCT GCG G -3'] and ITS4R [5'-TCC TCC GCT TAT TGA TAT GC-3'] (White et al. 1990). PCR reaction mixture (12.49 µl) consisted of 1 µl DNA mixed with 1× Phire PCR buffer, 0.2 mM each dNTPs, 0.2 µl Phire hot start II DNA polymerase enzyme, 0.1 mg /ml BSA, and 3% DMSO, 0.5M betaine and 5 pM of each primer. The PCR amplification was carried out in a PCR thermocycler (GeneAmp PCR system 9700, Applied Biosystems) with initial denaturation at 98 ^{0}C for 30 seconds, 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 55 ^{0}C for 50 seconds, elongation at 72^{0}C for 1 min and a final elongation step at 72 for 10 min. The amplified ITS PCR product was purified by PEG-NaCl precipitation.

Sequencing and Phylogenetic analysis

The purified PCR products were observed on 1.2% Agarose gel prepared in 0.5X TBE buffer having 0.5µg/ml EtBr. The gel was observed in a UV transilluminator after 1 to 2 hours. The sequencing was done on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc. Foster City, CA) as per the manufacturer's instructions. The sequence was deposited in NCBI under the accession no. OL757831.

The Tamura-Nei model and the Maximum Likelihood approach were used to infer the phylogenetic relationships (Tamura & Nei. 1993). The phylogenetic relationships of the taxa studied is assessed by bootstrap values estimated from 1000 replicates (Bitencourt et al. 2022). Branches that correspond to partitions that have been replicated with 50% of bootstrap replicates have collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Bitencourt et al. 2022). The initial tree(s) for the heuristic search were automatically generated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances evaluated using the Maximum Composite Likelihood (MCL) technique and then picking the topology with the highest log-likelihood value. There were 20 isolates treated in this study. The total number of base pairs in the final dataset was 4970. MEGA X was used to undertake the phylogenetic analysis (Kumar et al. 2018).

Results

Fusarium metavorans Al-Hatmi, S.A. Ahmed & de Hoog, in Al-Hatmi, Ahmed, van Diepeningen, Drogari-Apiranthitou, Verweij, Meis & Hoog, Medical Mycol. 56: S147 (2018)
Index Fungorum number: IF821742

Mycelium septate, branched. Sexual morph: Undetermined. Asexual morph: *Conidiophores* arise from the hyphae, hyaline, simple, bearing conidia. *Conidia* hyaline, phialosporous, of two kinds: macroconidia and microconidia. Macroconidia comprise slightly curved apical cells, 2 cylindrical central cells, often slightly curved on one side, and hooked foot cells, usually 3–4-celled and 16.32–53.04 μm (mean 34.68 μm, n = 20) in length and 2.04–4.08 μm (mean 3.06 μm, n = 20) in diameter; Microconidia cylindrical, 1–2-celled, 4.08–20.40 μm (mean 12.24 μm, n = 20) in length and 2.04 – 4.08 μm (mean 3.06 μm, n = 20) in diameter. *Chlamydospores* globose, present at hyphal tips (terminal) or within hyphae (intercalary), 5.8–13.4 μm (9.6 μm) in diameter (Fig. 1C-E).

Cultural characteristics – Colonies on PDA medium were white cottony with aerial mycelium. Due to the release of particular pigment exudation by the fungus in the culture medium, the colony's reverse color shows a gradient from light yellow at the periphery to yellowish-orange at the center. (Fig. 1 A-B)

Material examined – India, Jammu and Kashmir UT, Baramulla District, 34º03'N, 74º23'E, in temperate forest soil, 21 August 2021, M.A. Malik, living culture (HNY. KASH-2639).

GenBank Accession number – ITS: OL757831

Phylogenetic analysis
The reference sequences were acquired from GenBank after sequence analysis using the BLAST homology search against the NCBI database. The phylogenetic tree was rooted with *Fusarium staphyleae* strain (AF 178423.1). The sequences produced during the current investigation were compared to a data collection of 20 ITS rDNA nucleotide sequences from NCBI based on blast search. It was quite evident from phylogenetic analysis (Fig. 2) that *Fusarium metavorans* showed close genetic relatedness with other species of *Fusarium* such as *Fusarium metavorans* (NR 165517.1), *Fusarium neerlandicum* (NR 173438.1), *Fusarium ornamentatum* (NR 160126.1), *Fusarium witzenhausenene* (NR 172276.1), *Fusarium tokinense* (NR 170733.1), *Fusarium turanense* (NR 165843.1), *Fusarium suttonianum* (NR 172216.1), *Fusarium sp.* (GU 170640.1), *Fusarium sp.* (KT 313633.1), *Fusarium lichkenicola* (NR 173410.1), *Fusarium protoensiforme* DQ 094313.1), *Fusarium cucerbiticola* (DQ 094302.1), *Fusarium citricola* (NR 172265.1), *Fusarium buharicum* (NR 173416.1), *Fusarium acutatum* (NR 111142.1), *Fusarium concentricum* (NR 111886.1), *Fusarium burgessii* (NR 172292.1), and *Fusarium algeriense* (NR 158423.1).

Note – The morphological characteristics of *Fusarium metavorans* fit into the genus *Fusarium* by having white cottony, and septate, branched aerial mycelium, bearing conidia. Conidia are hyaline, phialosporous and of two kinds: macroconidia and microconidia. Macroconidia comprise slightly curved apical cells, 2 cylindrical central cells, often slightly curved on one side, and hooked foot cells, usually 3–4-celled. A multi-gene phylogeny generated herein indicates that *Fusarium metavorans* forms a strongly supported cluster (91%) to *F. neerlandicum*. However, *F. metavorans* is distinct from *F. neerlandicum* in having larger and narrower macroconidia (16.32–53.04 μm x 2.04–4.08 μm) and microconidia (4.08–20.40 μm x 2.04 – 4.08). This is the first report of *Fusarium metavorans* from the rhizosphere of *Digitalis purpurea*.

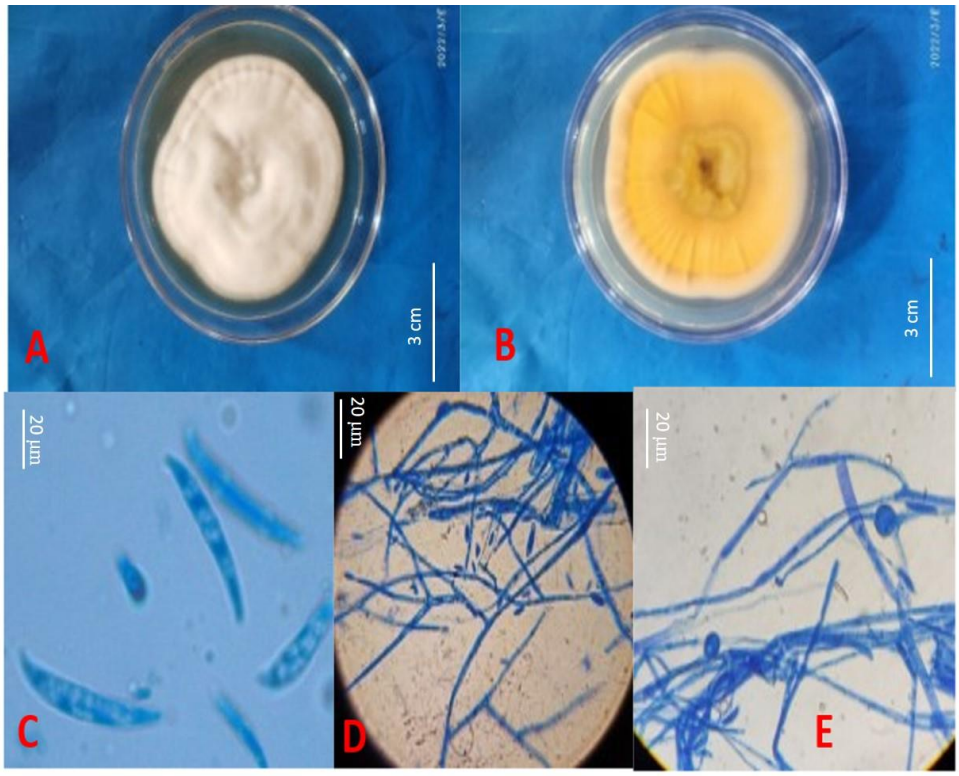

Fig.1 – *Fusarium metavorans.* (A) Colony characteristics on PDA. (B) reverse colony characteristics. (C) Macroconidia. (D) Mycelium and conidiophore bearing conidia at tip. (E) Chlamydospores.

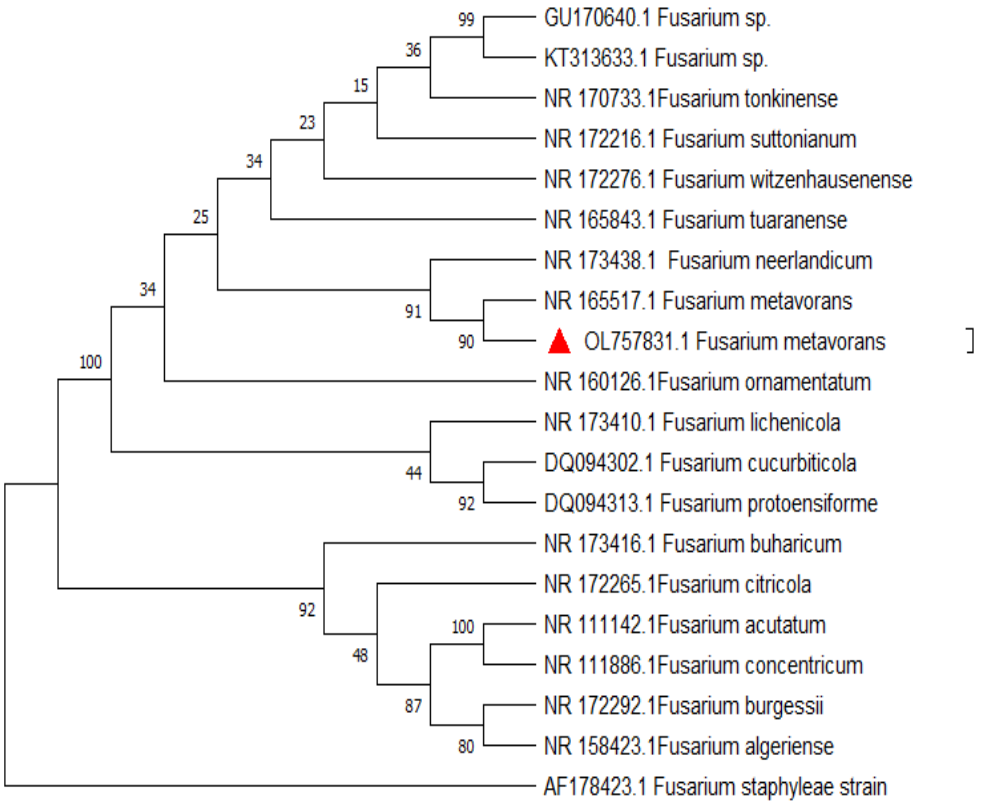

Fig. 2 - Phylogenetic placement of *Fusarium metavorans* as inferred from ITS by Neighbor-Joining method in MEGA-X with 1000 bootstrap replications.

Table 1 GenBank accession numbers used in the phylogenetic study of *Fusarium metavorans.*

S.No.	Species	NCBI Collection	References
1	*Fusarium* sp.	GU170640.1	Migheli et al. (2010).
2	*Fusarium* sp.	KT313633.1	Schroers et al. (2016).
3	*Fusarium tonkinense*	NR_170733.1	Sandoval-Denis et al. (2018).
4	*Fusarium suttonianum*	NR_172216.1	Zhang et al. (2006).
5	*Fusarium witzenhausenense*	NR_172276.1	Šišić et al. (2018).
6	*Fusarium tuaranense*	NR_165843.1	Kasson et al. (2013).
7	*Neocosmospora neerlandica*	NR_173438.1	Crous et al. (2021).
8	*Fusarium metavorans*	NR_165517.1	Al-Hatmi et al. (2018).
9	*Fusarium metavorans*	OL757831.1	Malik et al. (2021).
10	*Fusarium ornamentatum*	NR_160126.1	Vu,D et al. (2018).
11	*Fusarium lichenicola*	NR_173410.1	Unpublished. (2019).
12	*Fusarium cucurbiticola*	DQ094302.1	Zhang et al. (2006).
13	*Fusarium protoensiforme*	DQ094313.1	Zhang et al. (2006).
14	*Fusarium buharicum*	NR_173416.1	Vu et al. (2018).
15	*Fusarium citricola*	NR_172265.1	Sandoval-Denis et al. (2018).
16	*Fusarium acutatum*	NR_111142.1	Schoch et al. (2014).
17	*Fusarium concentricum*	NR_111886.1	Schoch et al. (2014).
18	*Fusarium burgessii*	NR_172292.1	Vu et al. (2018).
19	*Fusarium algeriense*	NR_158423.1	Laraba et al. (2017).
20	*Fusarium staphyleae strain*	AF178423.1	Unpublished (1999).

Discussion

The findings of this study confirmed the presence of *Fusarium metavorans* in the rhizosphere of *Digitalis purpurea*. Literature surveys also revealed that the species has a global distribution and a wide ecological amplitude, also including strains from soil and agents of opportunistic plant diseases, thus supporting our identification of the fungus from the rhizosphere of the medicinally important plant (De Hoog & Guarro 1995, Herr et al. 2016). The pathogenicity of the isolated fungus, however, was not assessed during this study. Species belonging to the genus *Fusarium* were isolated and identified from the rhizospheric soil of *Digitalis purpurea* from Kashmir Himalaya, India, using molecular identification methods as well as cultural and microscopic characteristics.

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Competing interests

There is no conflict of interest among the authors.

Accessibility of data

Accession no. OL757831 Voucher specimen No. 2639 (KASH) Herbarium, Centre for Biodiversity and Taxonomy, University of Kashmir, 21/08/2021, Gulmarg Kashmir. India,

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