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Metacordyceps dhauladharensis sp. nov., a new entomopathogenic fungus from India

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Abstract: This paper describes a new species of Clavicipitaceae fungi belonging to the genus *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, defined as *Metacordyceps dhauladharensis* sp. nov., from India. The diagnostic features of this new species are stipitate stromata of brownish color and an elongated fertile head on a Hymenopteran host. The perithecium size of this species was measured as 152–257 × 65–72 µm, and the asci were measured as 30–48 µm in length and 2.5 µm in breadth. Based on molecular studies, the sequence of 469 bp of this species showed only 86% homology with *Metacordyceps indigotica* (Kobayasi & Shimizu) Kepler, G.H. Sung & Spatafora.

Key words: Entomopathogenic fungi, *Metacordyceps*, ITS1-5.8S-ITS2, new species

1. Introduction

Entomopathogenic fungi are a group of insect pathogens. They are represented by 100 orders and 700 species around the world (Roberts, 1989). These fungi belong to the phyla Glomeromycota and Ascomycota with the orders Entomophthorales and Hypocreales, respectively (Hibbett et al., 2007; Sung et al., 2007). The genus *Cordyceps* Fr. includes entomopathogenic fungi belonging to the phylum Ascomycota and the order Hypocreales. This genus is represented by more than 400 species around the world (Kobayasi, 1982; Stensrud et al., 2005). Species of this genus have international distribution and a wide host range, from 10 orders of arthropods to the truffle-like genus *Elaphomyces* Nees. The taxonomic features of this genus are clavate to capitate stromata, production of superficial to immersed perithecia, cylindrical asci, thickened ascus apices, and filiform ascospores or partspores (Mains, 1958; Kobayasi, 1982; Rossman et al., 1999; Hywel-Jones, 2002). According to the recent classification of *Cordyceps* Fr., three subgenera (*C.* subg. *cordyceps*, *C.* subg. *Ophiocordyceps*, and *C.* subg. *neocordyceps*) have been raised based on the arrangement of perithecia morphology of asci, ascospores or part-spores, and molecular studies according to the phylogenetic placement of *Cordyceps taii* Z.Q. Liang & A.Y. Liu (Sung et al., 2007).

The genus *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora has been erected from the genus *Cordyceps* Fr. along with three other genera, namely *Cordyceps* Fr., *Ophiocordyceps* Petch, and *Elaphocordyceps* G.H. Sung & Spatafora, based on molecular phylogeny (Sung et al., 2007, 2010). *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora includes species with solitary or several stromata that are simple or branched; stipe that is fleshy or tough, whitish, greenish-yellow to greenish, and cylindrical to enlarging in the fertile part; perithecia that are partially or completely immersed in stromata, ordinal or oblique in arrangement; cylindrical ascospores; and multiseptate (Sung et al., 2007). Based on molecular phylogeny, it was found that the clade *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora is phylogentically associated with *Metarhizium* Sorokin and *Pochonia* Bat.& O.M. Fonseca (Sung et al., 2007).

There is a wide range of entomopathogenic fungi that are still hidden in the Himalayan regions of India, Nepal, Bhutan, and China. This new collection was made in the beginning of the winter season during frequent and exhaustive surveys in the Dhauladhar Mountains (a southern branch of the main outer Himalayan chain of mountains, about 2000–2200 m high). The collection was

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studied and taxonomically compared with other species with the help of authenticated literature and Mycobank descriptions.

2. Materials and methods

2.1. Collection and culturing

Six infected insect samples were collected from the forest of the Dhauladhar Mountains (2200 m altitude) in India. This fungus was found to be growing on the Hymenopteran insect body, which was deeply buried into the soil. After noting the details pertaining to morphology and habitat, culturing of the fungus was performed with the tissue culture technique (Chang and Quimio, 1982). The freshly infected insect body was washed thoroughly 4–5 times with water, dipped into 70% ethanol for 30 s, and then rinsed 3–4 times with sterile distilled water. The sterilized fruiting body was cut into small sections and incubated on potato dextrose agar (SD Fine, India) at 25 °C. The purified cultures were maintained at 25 °C.

2.2. Classical and molecular taxonomy

Macroscopic examinations were carried out on fresh specimens in the field. After culturing, the specimen was vacuum-dried at constant temperature (45 °C). Microscopic characters were studied in freehand sections mounted in 5% KOH and stained with 1% congo red (Sigma–Aldrich, USA). Taxonomical details were noted under high power lens (1000×). Color terminology in the description is based on the Methuen Handbook of Colour (Kornerup and Wanscher, 1978).

For the molecular study, 5-day-old mycelium was scraped from the petri dishes, frozen in liquid nitrogen, and ground to a fine powder. Genomic DNA was extracted using Qiagen Plant DNeasy Kit (Sigma–Aldrich, USA). The amplification of ITS1-5.8S-ITS2 gene region was achieved using primer pairs of ITS1 and ITS4 (White et al., 1990). The PCR reaction was performed in 50 µL total reaction volume including 50 ng of genomic DNA, 10 pmol of each primer, 0.5 mM dNTPs, 1X PCR buffer with 1.5 mM MgCl2, and 3 U *Taq* polymerase (Sigma–Aldrich, USA). The thermo-cycling conditions consisted of initial denaturation at 94 °C for 5 min, followed by 35 amplification cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 8 min. The sequence of the PCR products was determined by employing the ABI Prism Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequence was analyzed using the gapped BLASTn (Tamura et al., 2007) search algorithm aligned to the nearest neighbors. The evolutionary distances were calculated with Mega 4 software package version 1.3b (Yves Van de Peer, University of Antwerp, 1994, 1998) using Kimura 2 parameter, after aligning the sequences with ClustalW. Sequences of reference strain were obtained from the tree with NCBI GenBank.

3. Results

***Metacordyceps dhauladharensis* Sapan sp. nov.**

Type locality and habitat: Sample was collected from Dhauladhar Mountain of sub-Himalayan range at an altitude of 2200 m (above mean sea level) in mixed forest (*Pinus*, *Cedrus*, and *Quercus*).

Diagnosis: *M. dhauladharensis* was characterized as having 2–3 stromata arising from Hymenopteran insect body. Stromata consisted of brownish stipe and whitish pruinose head. Taxonomically, this species was characterized as having very small perithecium and asci.

Description: Two to three stromata attached to the body of Hymenoptera insect (body size 22–30 mm) with white cord-like mycelium, buried into the soil (2–3 cm) and covered with whitish mycelium. Stroma was fleshy when it was young and became hard at maturity. Stroma arose from the head and abdomen regions of the host insect. The stroma was measured as 50–65 mm in length and 10–20 mm in breadth. Fertile head was cylindrical, branched, with lateral branches at the apex region. Apex region impart pruinose appearance and whitish with 0.6 cm length. Stipe cylindrical, 40–50 mm in length, and brownish white with orange tinge. Scales were not observed on the stipe. Perithecia were partially immersed, hyaline-walled, globose to subglobose, obliquely arranged, measuring 152–257 µm in length and 65–72 µm in breadth. Ostioles were not visible on the outer surface. Asci with distinct cap, elongated, cylindrical, up to 30–48 × 2.5 µm, and intermingled in perithecium.

Ascospores were measured as (2–)2.7–5(–6.5) µm in length and 0.6–1.0(–1.2) µm in breadth. Conidia were measured as 3.7–8 × 0.7–1.0 µm in size. They were hyaline and cylindrical filled with granular content (Figures 1 and 2).

Etymology: The species was named on the basis of collection locality (Dhauladhar Mountains).

Collection examined (holotype): Dharamshala, forest of Dhauladhar Mountain (2200 m), Himachal Pradesh, Sapan Kumar, India.

Deposition numbers: Specimen has been deposited at Herbarium, Department of Botany, Punjab University, Chandigarh (Punjab), India. Mycobank no. MB807936, Genbank (ITS sequence) accession no. KJ179838.

Associated host: Sample was found to be associated with adult hymenoptera insect found buried in the soil. The entire insect body was covered with whitish mycelium.

Figure 1. **A.** *M. dhauladharensis* in wild habitat, **B.** Unearthed stromata associated with hymenopteran insect, **C.** Microphotograph of perithecia, **D.** Dividing conidia, **E.** Conidiogenesis, **F.** Asci, **G.** Mycelium.

Distribution: This species was found to be distributed at 32.238602°N, 76.323878°E in the Himalayan zone.

Phylogeny: Based on the sequence of ITS1-5.8S-ITS2 gene region, which was approximately 700 bp. The fungus showed 86% identity with *Metacordyceps indigotica* (Kobayasi & Shimizu) Kepler, G.H. Sung & Spatafora (Table 1). The dendrogram was constructed with MEGA 4.0 software (Figure 3). All positions containing gaps and missing data were eliminated. Presently described species is different from *M. indigotica* in morphology, taxonomical details, and host insect, as described by Kepler et al. (2012). Morphological features such as stromata

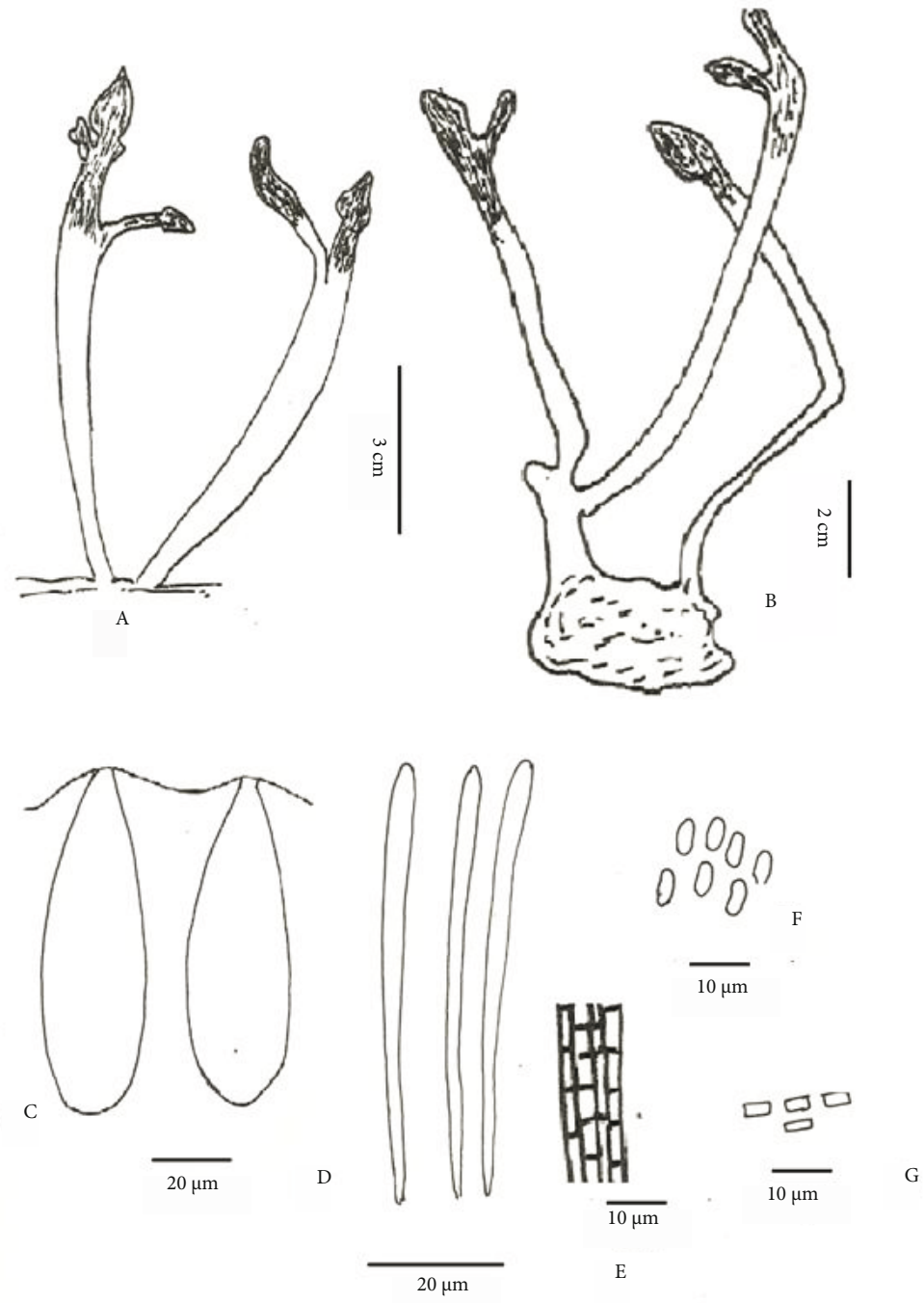

Figure 2. **A.** Stromata, **B.** Unearthed stromata, **C.** Perithecia, **D.** Asci, **E.** Apical part of asci, **F.** Conidia. **G.** Partspores.

Table 1. Results of five closely related species with percent identity and accession numbers.

Species name	Percent identity	Accession number
Metacordyceps indigotica CGAC2702-B	86	JN180464.1
M. indigotica isolate CGAC2702-A	86	JN180463.1
M. taii isolate 36	86	KJ021191.1
M. taii clone DS1A2	86	JX177480.1
M. taii clone DS1A2	86	JX177478.1

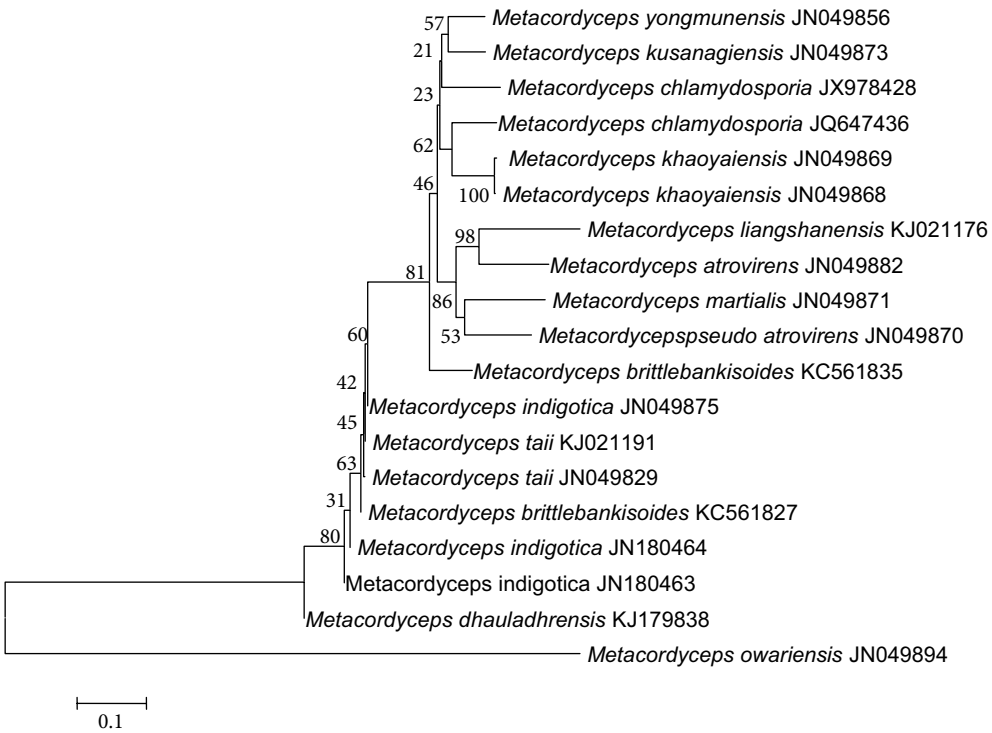

Figure 3. Phylogenetic evolutionary tree of *M. dhauladharensis* with related species. The numbers on the nodes indicate how often (number of times, %) the species to the right are grouped together in bootstrap values (1000). Bar 0.1 substitutions per site.

shape, color, and stipe length of *M. dhauladharensis* are somewhat similar to those of *M. owariensis* (Kobayasi) Kepler, G.H. Sung, & Spatafora, but differ in taxonomical details. Perithecia in *M. dhauladharensis* (152–257 × 65–72 µm) are smaller than those in *M. owariensis* (Kobayasi) Kepler, G.H. Sung, & Spatafora (460–530 × 200–270 µm). Asci in *M. dhauladharensis* (4–4.3 µm) were observed as narrower than in *M. owariensis* (Kobayasi) Kepler, G.H. Sung, and Spatafora (4–4.3 µm) (Kepler et al., 2012). Perithecium of this species was found to be very small compared to that of *M. guniujiangensis* C.R. Li, B. Huang, M.Z. Fan & Z.Z. Li, in Li, Huang, Fan, Lin & Li (640–770 × 240–320 µm). Another allied species of *M. dhauladharensis* is *M. taii* (Z.Q. Liang & A.Y. Liu) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, which possesses cylindrical, yellow-green stromata measuring 20–35 × 2–5 µm and large perithecium measuring 767–940 × 247–354 µm. On the basis of stroma length, this species appears close to *Cordyceps owariensis* f. *viridescens* Uchiy. & Udagawa (Uchiyama and Udagawa, 2002) and *Cordyceps brittlebankisoides* Zuo Y. Liu, Z.Q. Liang, Whalley, Y.J. Yao & A.Y. Liu (Liu et al., 2001). Length of perithecium in *M. dhauladharensis* was observed as much smaller compared to these two species. Details of morphology and perithecium size of present species and its allied species are given in Table 2.

Table 2. Stromatal morphology and perithecium length of *M. dhauladharensis* and allied species (Kobayasi 1982).

Species	Morphology of stromata	Host order	Perithecium length (μm)
Cordyceps ctenocephala	4–6 mm long, orange-yellow	Hemiptera	630–900
C. militaris	25–45 mm long, orange-yellow	Lepidoptera	450–670
C. typhulaeformis	20 mm long, flesh-colored	Hemiptera	300–400
C. langloisii	15–30 mm long, reddish purple	Hymenoptera	250–300
C. scarabaeicola	30–40 mm long, pale yellow	Diptera	275–350
C. kyushuensis	20–70 mm long, orange-yellow	Lepidoptera	450–500
C. doiana	30 mm long, ochraceous	Hymenoptera	250
C. cylindrical	25 mm high, pale yellow	Araneae	1200
C. sinclairii	20 mm high, pale ochraceous	Hemiptera	270
C. salebrosa	35–40 mm, light cream-yellow	Coleoptera	840–1200
C. trinidadensis	2 mm long, light brown	Orthoptera	250–300
C. ridleyi	3–4 mm long, dark brown	Hymenoptera	260–300
C. coccinea	3–7 mm high, red	Coleoptera	180–210
C. sulphurea	40–50 mm long, sulphur yellow	Lepidoptera	220–250
C. gemella	5–8 mm high, red-brown	Coleoptera	390–560
C. elongate	30 mm high, fertile part orange	Lepidoptera	250–300
C. sobolifera	20–80 mm high, pale brown	Hemiptera	500–600
C. nikkoensis	50–120 mm long, ochraceous	Lepidoptera	460–500
C. vinosa	15–40 mm long, purplish red	Lepidoptera	260–360
C. pseudoinsignis	20–30 mm long, yellow ochraceous	Coleoptera	900–1100
C. brittlebankisoides	30–50 mm long, pale green	Coleoptera	188–313
Metacordyceps owariensis	Grayish–green or dark herbage green, acute, white, glabrous sterile tip	Homoptera	440–640
M. guniujiangensis	Dark green, yellow, acute, glabrous sterile tip	Homoptera	640–770
M. dhauladharensis	50–65 mm long, brownish stipe and whitish pruinose head	Hymenoptera	152–257

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