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First report on occurrence of *Clonostachys* in cave ecosystem from India

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Present paper deals with presence of interesting fungi in cave ecosystem. Microscopic fungi were found associated with various substrates in caves, such as sediments, vermiculations, soil and decaying organic materials. During our study an interesting species of *Clonostachys* was isolated in addition to other known fungi. Literature reveals that researches on microfungi of cave ecosystem and other related aspects has received much attention in recent past, but to our understanding this is the first report unveiling the presence of *Clonostachys rosea*. The identity of this interesting fungus was confirmed by morphological and sequence analysis of ITS gene region and their phylogenetic analysis. During study this fungus was recorded from limestone, silica karst and soil of two temple caves *viz.,* Pandav and Lakhmandal of Doon Valley of Uttarakhand Himalayas having its own historical importance.

Key words: Cave microfungi, Doon Valley, Himalaya, rDNA, Taxonomy

INTRODUCTION

Doon valley is a wide long valley, within the Shivalik hills in the lesser Himalayas, extending to the Uttarakhand, Himachal Pradesh and Haryana states. Within the valley lies the city of Dehradun, the capital of Uttarakhand state. Cave ecosystems are extreme environments characterized to possess very low nutrients level. (Pederson, 2000). Within a cave ecosystem, there are different micro-environments inhabited by plethora of microorganisms, which are influenced by specific conditions, like humidity, stable temperatures, nutrients and pH (Mulec *et al*. 2002).

A large number of these heterotrophic and chemolithotropic microorganisms in caves are involved in lithogenic processes. (Canaveras *et al*. 2006; Engel *et al*. 2004; Mulec *et al*. 2007).

Being an integral part of cave ecosystems, fungi grow on various substrates in caves. Their widespread distribution contributes to their important role in the feeding strategies of cave fauna. Handful of studies report the microfungi of cave environment. Man *et al.* (2015) have reported for the first-time the presence of fungal communities its functions in cave ecosystems in a natural pristine cave of central China. Recent work on diversity of fungal species in Mandeepkhol cave, Chhattisgarh report 54 species belonging to 16 genera and two mycelia sterilia (Karkun *et al*. 2012). In India, especially in Uttarakhand, little information is available on microfungi of cave ecosystem and/or environment. With this background, the present study was conducted.

MATERIALS AND METHODS

Sample collection

Two temple caves of vedic history *viz.,* Lakhamandal and Pandav caves were surveyed during present study. Soil and limestone karst samples (100 g) were collected separately in sterile polythene bags. Collected samples were brought

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to the laboratory and subjected to the isolation of fungi following serial dilution method (Waksman, 1916; Waksman and Fred, 1922) using PDA and MEA media. Pure cultures of the fungi were obtained by single spore culture and sometimes by hyphal tipping method (Warcup, 1955a).

Isolation of Fungi

Serial dilution method was used. Briefly, the stock solution was prepared by mixing 1 gm of sample in 10 ml sterile distilled water. Then dilutions from stock solution were made up to 10^{-7}. Then 100µl from dilution (10^{-3}, 10^{-5}, 10^{-7}) was used as inoculum on two different culture media, Malt Extract Agar (MEA), and Potato Dextrose Agar (PDA). After 3-5 days of incubation at 25ºC, plates were observed and colony exhibiting different morphological characters were selected, purified, and transferred onto fresh PDA slants for further study. Three sets of each pure culture were prepared. The One set was subjected to identification, the second set was preserved in laboratory uses, and the third set was meant to deposit and accession in the repository.

Morphological identification and characterization

The pure cultures obtained were segregated into two parts, sporulating and non-sporulating cultures. Preliminary identification of well sporulating cultures was done based on morph-ological characterization using standard-literature/ keys, while non-sporulating/sterile culture was subjected to further processing of inducing sporulation. The pure and interesting cultures isolated dealt in this study were inoculated on different media such as potato dextrose agar (PDA), and malt extract agar (MEA), to study and record their morphological characters. After 14 days of incubation at 25ºC, cultural characteristics were studied. Methuen handbook of color was used for recording the color of the colony (Kornerup and Wanscher, 1978). Pure cultures of both the taxa were deposited in NFCCI (National Fungal Culture Collection of India), Pune (NFCCI 5032 and NFCCI 5034).

Molecular Identification: DNA extraction

The protocol by Aamir *et al*. (2015) was followed for fungal genomic DNA extraction. Briefly, fungal mycelia from the seven days old PDA culture plate were scrapped and taken into a DNA extraction tube containing ceramic pestle and glass beads. 1 ml of lysis buffer containing 100 mM Tris–HCl of pH 8.0, 50 mM EDTA, and 3% SDS was then added into the tube and subjected to homogenization at 6 M/S for 60 seconds using FastPrep®–24 tissue homogenizer (MP Biomedicals, USA). The resultant lysed and homogenous mixture was subjected to centrifugation at 13,000 rpm for 10 minutes. The supernatant containing genomic DNA was collected into a new sterile microcentrifuge tube. The purification of genomic DNA was done by adding an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) followed by centrifugation at 13,000 rpm for 10 minutes. The upper aqueous part was then collected into a 1.5 ml microcentrifuge tube, and precipitation of the DNA was done using pre-chilled Isopropanol. Pelleted DNA was then washed with 70% ethanol by centrifugation (12,000 rpm for 5 minutes). The resultant DNA pellet was air-dried and dissolved in TE buffer (1x).

Lastly, 2 µl of RNA-se A (20 mg/ml) was added and incubated at 37 ^{0}C for one hour.

Polymerase chain reaction

Internal transcribed spacer (ITS), of ribosomal DNA (rDNA) were amplified from the genomic DNA by Polymerase Chain Reaction (PCR) using Applied Biosystems ProFlex PCR machine (Table1). Primer pairs used to amplify ITS region (ITS4 & ITS5), and amplification condions are listed in Table 1 (White *et al.,* 1990. Using Favor PrepTM PCR Purification kit (Favorgen Biotech Corporation, Taiwan), amplified PCR products were purified and then used for sequencing by an ABI Avant 3100 automated DNA sequencer and BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). Finally, obtained sequences of ITS, region of rDNA deposited in the NCBI nucleotide sequence database (GenBank Acc. No. ITS– OM494340 (NFCCI 5032), and OM494342 (NFCCI 5034).

Sequence alignment and phylogenetic analysis

To determine the phylogenetic relationship of the present isolates, ITS gene region was used to compare the present isolates with already known taxa of *Clonostachys* genus. The sequences of the related strains were retrieved from NCBI. A total of 23 isolates of *Clonostachys* genus were used in the analysis and were aligned along with

sequences of *Clonostachys rosea* NFCCI 5032 and *Clonostachys rosea* NFCCI 5034. *Dialonectria episphaeria* CBS 125494 was selected as the outgroup taxon. The chosen strains used in the construction of the phylogenetic tree along with their accession numbers and other related details are listed in Table 2. Sequences were aligned using MAFFT v. 6.864b (Katoh and Standley, 2013). The alignments were checked and adjusted manually using Aliview (Larsson, 2014). The best substitution model was found using Model Finder (Kalyaanamoorthy *et al*. 2017). Further, windows version IQ-tree tool v.1.6.11 (Nguyen *et al*.2015) was used to reconstruct the phylogenetic tree. The reliability of the reconstructed branches was assessed by Boot-strap analyses of 1000 replicates. The constructed phylogenetic tree was visualized in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS AND DISCUSSION

Taxonomy

Clonostachys rosea (Link) Schroers, Samuels, Seifert & Gams (1999). Mycologia 91 (2): 369 (1999). (Fig.1)

Colonies on MEA white to buff, creamy, 45 mm diam in 12 days. Reverse buff, floccose. Primary conidiophores arising from superficial hyphae, densely penicillate which bears slimy or gloeosporic one celled conidia, erect, produced from aerial or submerged hyphae or from hyphal bundles, divergent, verticillately branched, septate, smooth walled, variable in size up to 450 × 2.5 μm. Secondary conidiophores: densely penicillate, dichotomously branched, septate, smooth walled, hyaline up to 112.5 × 3.12 μm. Primary phialides convergent and divergent, terminal to intercalary, mostly 1-4 in a group, aculeate (slender gradually decreasing towards length), variable in size, smooth walled, hyaline, 25-30 × 2.5 μm. Secondary phialides on secondary conidiophores: convergent, up to 5 in a group,variable in size, ampulliform, smooth walled, hyaline, 12.5 -15 × 2-2.5 μm. Conidia produced in gloeosporic mass, abundant, symmetrical to asymmetrical (navicular to broadly ovoid to ovoid), smooth walled, hyaline, 3.75 -6.25 × 2.5 μm.

Material examined: INDIA, Uttarakhand, Doon Valley, Lakhamandal and Pandav caves, from the soil,.7. Nov. 202, Meena Thapliyal & S. Guleri, living culture, NFCCI 5032, & 5034.
GenBank numbers: ITS = OM494340 (NFCCI 5032); OM494342 (NFCCI 5034)

Phylogenetic analyses : Based on white to buff, creamy colony characters and verticillate as well as penicillate conidiophores bearing single celled conidia. Both the isolates i.e NFCCI 5032 and NFCCI 5034 were tentatively placed under the genus *Clonostachys*. On megablast analysis, ITS sequence of NFCCI 5032 and NFCCI 5034 showed nearly 99% identity with known taxa; *Clonostachys rosea* strain CBS 126933 (540/541; 99.8%), strain CBS 127294 (543/544 bp 99.8%) and with type *Clonostachys rosea* f. *catenulata* CBS 154.27 541/544 bp (99.44%). Therefore, identity of both the taxa was confirmed as *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams.

The ITS sequence alignment was used to confirm the resolution of the present isolate. TIM2e+G4 was found to be the best-fit model of 286 models tested and was chosen on the basis of the Bayesian Information Criterion (BIC). The phylogeny was inferred by using the Maximum Likelihood Method based on the model mentioned above. Log-likelihood of consensus tree was -3167.800. Tree branches were tested based on 1000 ultrafast bootstrap (UFBoot) support replicates as well as with SH-like approximate likelihood ratio test (SH-like aLRT) with 1000 replicates. Phylogenetic analysis using ITS nested the present isolate in *Clonosyachys rosea* clade supported with goodSH-like aLRT, and ultrafast bootstrap (UFBoot).

During our study, we isolated the present taxon from a soil sample. The presence of microbes in caves had been reported earlier. It was only in 2015 that the presence of fungal communities and its

Table 1: Polymerase chain reaction (PCR) conditions used for amplification of ITS, rDNA

	PCR conditions					
PCR	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
ITS	94ºC for 5 min.	94ºC for 1 min.	48ºC for 30 sec.	72ºC for 1 min.	72ºC for 7 min.	30

Table 2: Taxa used in the phylogenetic analyses and their GenBank accession numbers

Species	Culture Accession No.	ITS	Location	Host/Substrate
Clonostachys rosea	NFCCI 5032	OM494340	Uttarakhand, India	Soil and karst limestone
Clonostachys rosea	NFCCI 5034	OM494342	Uttarakhand, India	Soil and karst limestone
Clonostachys rosea	CBS 126933	MH864340	USA: Wyoming	-
Clonostachys intermedia	CBS 508.82	NR_137652	Netherlands	Agricultural soil
Clonostachys miodochialis	CBS 997.69	NR_137649	Netherlands	Agricultural soil
Clonostachys rosea	CBS 376.55	MH869057	United States of America	*Acer palmatum*
Clonostachys ralfsii	CBS 127880	MH864738	Portugal	*Laurus novocanariensis*
Clonostachys rosea	CBS 127642	MH864650	USA: North Carolina	-
Clonostachys rosea	CBS 127294	MH864507	USA: Kansas	-
Clonostachys rosea	CBS 127143	MH864436	USA: Iowa	-
Clonostachys solani	CBS 102418	MH862790	Netherlands	Bark
Clonostachys solani	CBS 697.88	MH862150	Germany	Rotten trunk
Clonostachys rosea	CBS 710.86	MH862010	Netherlands	Soil
Clonostachys rosea	CBS 907.72B	MH860627	Ukraine	Forest soil
Clonostachys rosea	CBS 148.72	MH860422	Ukraine	Soil
Clonostachys compactiuscula	CBS 653.70	MH859887	Netherlands	Decaying Wood
Clonostachys kowhai	CBS 461.95	AF358250	New Zealand	*Sophora microphylla*, bark
Clonostachys buxi	CBS 696.93	KM231840	France	*Buxus sempervirens*
Clonostachys setosa	CBS 917.97	MH862683	Puerto Rico	Wet, decayed twigs on ground
Clonostachys rogersoniana	CBS 582.89	AF210691	Brazil	Rain forest soil
Clonostachys chlorina	CBS_287.90	MH862212	Brazil	Soil under *Theobroma cacao*
Clonostachys phyllophila	CBS_921.97	AF210664	France	*Viscum album*, leaves, fallen plant
Clonostachys phyllophila	CBS_685.96	AF210663	Cuba	
Dialonectria episphaeria	CBS_125494	MH863609	Canada	Old ascomycete stromata
Clonostachys candelabrum	CBS_504.67	MH859044	Netherlands	Wheat field soil

functions in cave ecosystems in a natural pristine cave of central China were reported (Man *et al.* 2015).In India, Karkun *et al*. (2012) worked on the diversity of fungal species in Mandeepkhol cave, Chhattisgarh and reported 54 species belonging to 16 genera and two mycelia sterilia.

In the present study, out of several isolates studied, two isolates were showing initially white to buff colony morphology on MEA. Due to limitations of morphological morphological identification, both the selected isolates were subjected to multigene sequencing and phylogenetic analysis. Overall, the study revealed that the taxa have a similarity (strong bootstrap value 99.8%) with *Clonostachys rosea* (Link) Schroers, Samuels, Seifert and Gams (1999). The same is illustrated and described here based on morphology and molecular phylogenetic analyses, which in our understanding turned out to be a first report as cave fungus from India.

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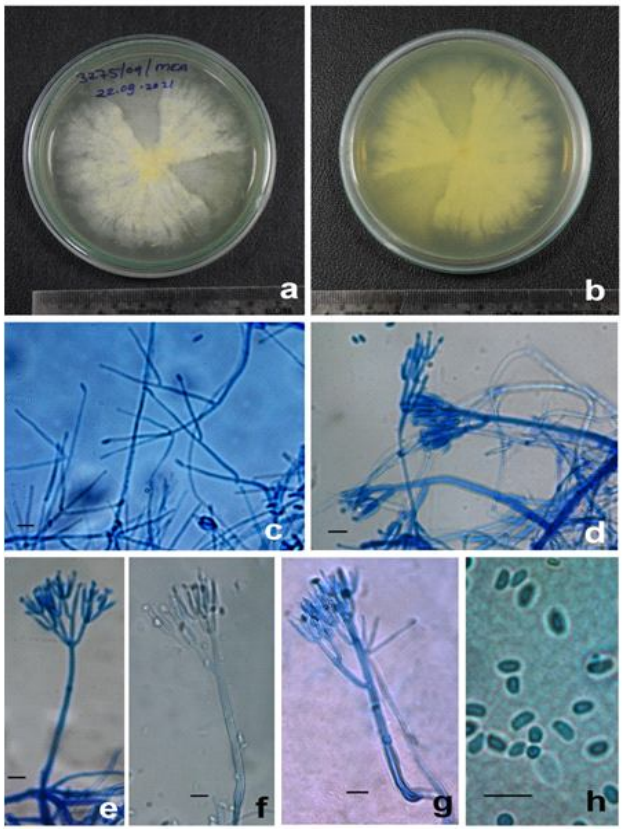

Fig. 1 : *Clonostachys rosea* **a.** Colony morphology on MEA (NFCCI 5034) after 14 days (Front view), **b**. Reverse view of colony on MEA. **c.**Verticillate like conidiophores with phialides, **d-g.** Penicillate conidiophores arising from vegetative hyphae and intact conidia,**h**. Single celled phialoconidia variable in shape. Scale bars (c-g) = 10 μm.

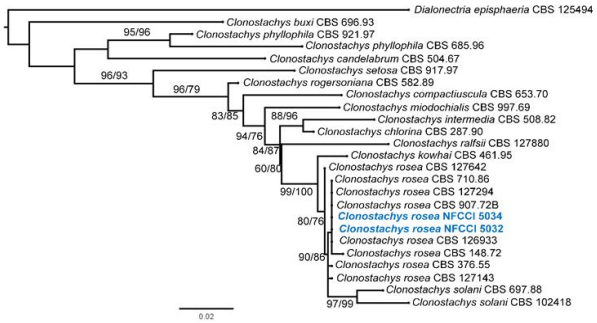

Fig. 2 : Molecular phylogenetic analysis by maximum-likelihood (ML) method based on ITS sequence data. Isolates (NFCCI 5032 and 5034) used in study are in blue bold. The tree is rooted to *Dialonectria episphaeria* CBS 125494.

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