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Full Length Article

A New Dacrymycetaceous Species, *Calocera himalayca* sp. nov. (Basidiomycota: Dacrymycetales) from Pakistan

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Abstract

While exploring biodiversity of mushrooms from Pakistan, *Calocera himalayca* was found growing on decaying wood in coniferous forests of Pakistan's Part of Himalaya. *C. himalayca* is very greasy in texture unlike the true coral fungi. Morphologically, it resembles with *C. viscosa* and often confused with some of the *Ramaria* species of coral fungi, but the greasy, viscid surface is an immediately obvious distinguishing feature of this species. The basidiomata were collected and characterized morpho-anatomically and rDNA was used to infer its phylogenetic topology/ placement with its allies. © 2015 Friends Science Publishers

Keywords: Amplification; Bright orange; Logs; Stipitate basidioma

Introduction

Genus *Calocera* (Fr.) Fr. is represented by 15 species worldwide (Kirk *et al.,* 2008). From Pakistan, only three species of this genus viz; *C. cornea* (Batsch) Fr., *C. stricta* Fr. and *C. viscosa* (Pers.) Fr., have been reported (Ahmad *et al.,* 1997). They are greasy in texture unlike the true coral fungi and often confused with some members of Gomphales (species of *Ramaria* Fr. ex Bonord. and *Clavulina* J. Schröt.), but the greasy, viscid surface is an immediately obvious distinguishing feature of this species. The order Dacrymycetales is one of the wood decaying group of fungi which are involved in the degradation of cellulose, hemicelluloses and lignin. They have mechanism of lignin degradation involving degradatory enzymes (ligninolytic enzymes, lignin Peroxidases, manganese Peroxidases, Laccases, aryl-alcohol Oxidases). These are also used in biological pulping, kraft pulp discoloration, decolorization of waste waters, coal solubilization, degradation of polystyrenes, bioremediation of toxic environmental pollutants, chlorinated organic compounds, polycyclic aromatic hydrocarbons, nitro-substituted compounds, dyes and other toxic compounds (Rajarathnam *et al*., 1998). Fungal inventories within the Himalayan Moist Temperate Forests (HMTF) of Pakistan revealed a new species of *Calocera* growing on decaying logs *Abies pindrow* Royle. This new species, *C. himalayca* is supported by molecular extraction and is described herein.

Materials and Methods

The sampling sites are located in the HMTF of Pakistan and show high basidiomycetes biodiversity. These forests are dominated by conifers (Khalid, 1998; Niazi, 2008). The temperature ranges from -4 to 25°C. Soil is loamy with gravels and rock stones of variable sizes. These conditions ideally favor the decomposition of rotting log. During the exploration of biodiversity of mushrooms, a species of *Calocera* was collected from Helipad, Khanspur-Ayubia found growing on *Abies pindrow* rotting logs. Field notes were prepared. This fungus was given a tentative number and vouchered. It was morphologically characterized following Reid (1974). Small portions from the hymenium (about 1 cm) were placed in 2% CTAB buffer in 1.5 ml eppendorff and kept at -20°C for further analysis. The collected specimen was dried with fan heater overnight and kept in vouchered Ziploc bags. The measurements of spores and other microscopic features were taken with micrometer and were drawn with the aid of a camera lucida. Basidiospores were observed at 1600×.

DNA Extraction

DNA was extracted by modified CTAB method following Gardes and Bruns (1993). The hymenial tissue was removed with sterile forceps and rinsed with sterile H_2O. The extraction was modified for silica emulsion binding and purification (Gene-Clean; Q-Biogene, Irvine, CA, USA).

Polymerase Chain Reaction and Sequencing

Polymerase chain reaction (PCR) was carried out following Gardes and Bruns (1993), using the fungus-specific ITS1F primer (CTTGGTCATTTAGAGGAAGT) and the

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eukaryotic ITS4 primer (TCCTCCGCTTATTGATATGC) to amplify the nuclear rDNA-ITS region. The hot-start enzyme JumpStart (Sigma, St Louis, MO, USA) was used to catalyse the PCR with 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, 40 s + 5 s per cycle at 72°C, and finishing with 5 min at 72°C. The PCR products were purified with QIAquick (Qiagen Inc., Valencia, CA, USA), sequenced bi-directionally using the reverse and forward primers and BigDye 3.1 on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and edited in sequencher 4.5 (Gene Codes, Ann Arbor, MI, USA) in Jodrell Laboratory, Royal Botanical Gardens, Kew, UK. DNA sequences were submitted to Basic Local Alignment Search Tool (BLAST) and used to query the nucleotide collection using default settings. DNA sequences of *C. himalayca* was submitted in GenBank and its phylogenetic position was inferred.

Alignment and Phylogenetic Analysis

Phylogenetic position of *C. himalayca* was confirmed by making tree using 30 rDNA ITS sequences including four (04) sequences obtained from *C. himalayca* from Pakistan. All the sequences were aligned and corrected manually by using Clustal W and Mega 5 programme used for making Maximum likelihood tree with 1000 bootstrapping. Percentage Identities were calculated using software DNA Star (DNA Star, Inc. 3801 Regent Street Madison, WI 53705 USA). Aligned sequences were then used for making phylogenetic tree.

Results

Enumeration of Taxon

Calocera himalayca sp. nov. Hanif and Khalid Fig. 1(A–C).

Etymology:

Calocera himalayca is named due to the holotype location in Himalayan Forests.

Stipitate and deep-rooted basidiomata, bright yellow to orange in color, dichotomously branched with pointed tips. Have frequent Probasidia, cylindrical to clavate, becoming bifurcate.

Morphoanatomical Characterization

Basidiomata stipitate, deep-rooted, 5–10 cm long, bright yellow when fresh becoming orange on drying, dichotomously branched, branches erect, terete, or compressed, with pointed tips. Hymenium amphigenous Probasidia cylindrical to clavate, becoming bifurcate, 40–50 x 5–6 µm. Basidiospores subglobose to reniform, hyaline, apiculum at the base, 1–septate, guttulate, 7.0–9.0 x 4.4–6.6 µm.

Habit and Habitat

On decaying *Abies pindrow* logs, in group of 2–4 basidiomata.

Material Examined

Pakistan, KPK, Aubia, Khanspur, Helipad, 34° 01′ 30.89″ N, 73° 25′ 18.78″E, elevation 1974 m, 26 Jul 2008, M. Hanif, MH2678.141 (LAH # MH2678.1 HOLOTYPE); KPK, Nathia Gali, Near Governor's House, 34° 04′ 18.18″ N, 73° 23′ 34.44″E, elevation 2408 m, 18 Aug 2009, M. Hanif, MH188902.288 (LAH # MH188902.2 PARATYPE). Punjab, Murree, 33° 54′ 25.60″ N, 73° 23′ 36.90″ E, elevation 2188 m, 11 Aug 2009, M. Hanif, MH1189.302 (LAH # MH0889.3 PARATYPE). Gilgit Baltistan, Fairy Meadows, 26 Aug 2010, M. Hanif, MH26810.287 (LAH # MH26810.4 PARATYPE).

Molecular Phylogenetic analysis

Calocera himalayca generated 209-219 bases long fragments when PCR products of rDNA-ITS were sequenced bidirectionally (Fig. 4). Initial BLAST analysis of all the isolates resulted in 98% similarity with *C. viscosa* and 95% query coverage (DQ 520102.1). rDNA-ITS sequences of all these 4 isolates of *C. himalayca* were identical to each other (Fig. 5). In order to investigate its molecular phylogenetic relatedness with the rDNA sequences of its related species deposited in the GenBank and with other sequences of its morphological allies, phylogenetic tree was constructed. Total 23 rDNA-ITS sequences were included along with 4 sequences of *C. himalayca* isolates from Pakistan, while *Xylaria* sp. was used as an out group. The aligned ITS1-5.8S-ITS2 dataset was 387 bases long, out of which 133 ambiguously aligned characters were excluded from the analysis. The alignment of 255 characters was used for further analyses. The maximum likelihood tree was constructed to find out molecular relatedness. In the alignment of 255 characters, 127 nucleotides were conserved, 92 were parsimony informative and 117 were variable.

Cladogram was divided into 2 major clades (Fig. 3). All the sequences belonging to order *Decrymycetales* were clustered together in clade I with strong bootstrap frequency (91%). All the isolates of *C. himalayca* grouped together within clade I with high bootstrap support (98%). Clade II composed of sequences of order *Gomphales*; morphological allies of genus *Calocera*. Isolates of *C. himalayca* from Pakistan shared 100% analyzed genetic characters with each other. These isolates shared 95.45% analyzed genetic characters with *C. viscosa* (DQ520102.1) and significantly differed genetically (3.0–5.2%). These isolates of *C. himalayca* shared 91% analyzed genetic characters and had 12% genetic divergence (Fig. 2) compared with *C. cornea* (AY789083.1). There were 9 polymorphic sites indicating insertions and deletions in alignment of 4 isolates of *C. himalayca* and *C. viscosa* (DQ520102.1) at positions 26, 31, 44, 168, 207, 210, 211, 212 and 219 (data not shown). Phylogenetic analysis and number of polymorphic sites confirm that *C. himalayca*

Table 1: rDNA sequences downloaded from GenBank for molecular characterization and phylogenetic analyses

Name of fungal species	Isolate/Voucher No.	Accession No.	County of origin
Calocera cornea	AFTOL-ID 438	AY789083.1	USA
Calocera himalayca	MH288	Unpublished	PAKISTAN
Calocera himalayca	MH287	Unpublished	PAKISTAN
Calocera himalayca	MH302	Unpublished	PAKISTAN
Calocera himalayca	MH141	Unpublished	PAKISTAN
Calocera sp.	KRCF731	AB374292.1	JAPAN
Calocera sp.	Wu9910-12	FJ195751.1	TAIWAN
Calocera sp.	ICMP 16998	GQ411508.1	NEW ZEALAND
Calocera viscosa	AFTOL-ID 1679	DQ520102.1	GERMANY
Cerinosterus luteoalbus	WRCF-AW12	AY618667.1	CANADA
Clavulina cf. amethystina	O 62152	EU862204.1	SPAIN
Clavulina cf. amethystina	PRM 896664	EU862203.1	SPAIN
Clavulina cf. amethystina	O 175524	EU862208.1	SPAIN
Clavulina cf. cristata	O 65398	EU862205.1	SPAIN
Clavulina cf. rugosa	O 67776	EU862207.1	SPAIN
Clavulina samuelsii	PDD:89881	GU222317.1	NEW ZEALAND
Dentocorticium sulphurellum	FP11801	JN165018.1	USA
Marasmius androsaceus	ZK24/08	FR717227.1	CZECH REPUBLIC
Marasmius androsaceus	NN008037	JN943605.1	USA
Ramaria botrytis	snf213	AF377055.1	USA
Ramaria formosa	OSC 1064203	EU525994.1	USA
Ramaria stricta	JMP0055	EU819419.1	USA
Xylaria hypoxylon	MH143	Unpublished	PAKISTAN

is a novel species, which nested with genus *Calocera* and *C. viscosa* appeared as its sister species.

Discussion

The taxonomic history of Dacrymycetalean genera has been reviewed by Oberwinkler (1993). Some studies discussing the phylogenetic relationship in this class have been published (Weiss and Oberwinkler 2001; Shirouzu *et al.,* 2007). Shirouzu *et al.* (2009) re-examined Dacrymycetous fungi in Japan using taxonomic studies along with molecular phylogenetic analyses. *Dacrymycetales*, initially established by Hennings (1898; as *Dacryomycetinieae*) and composed of the single family *Dacrymycetaceae,* which was introduced by Schröter (1889; as *Dacryomycetini*) including many genera. McNabb (1964, 1965a–e, 1966, 1973) re-examined the validity of described genera and finally recognised eight genera in the *Dacrymycetaceae* viz: *Calocera*, *Cerinomyces*, *Dacrymyces*, *Dacryopinax*, *Ditiola*, *Femsjonia*, *Guepiniopsis* and *Heterotextus*.

Genus *Calocera* of class Dacrymycetes represented by only 3 species in Pakistan viz; *C. cornea*, *C. viscosa* and *C. stricta* (Ahmad *et al.,* 1997). *Calocera himalayca* was first collected from Helipad, Khanspur, KPK growing on decaying wood of *Abies pindrow*. Initially this was confused with *C. viscosa*. When rDNA-ITS regions this species was amplified and analyzed, it looked different from *C. viscosa* already described from Pakistan phylogenetically as well. *Calocera himalayca* clustered within Clade Dacrymycetales for the present studies (Fig. 3). Members of class Dacrymycetes are known as jelly fungi characterized by imperforate parenthesomes and basidia that are usually branched. The present species, *C. himalayca,* is being described as a new addition, which is characterized by stipitate, deep-rooted dichotomously branched basidiomata, bright yellow to orange colour. This species looks morphologically very close to *C. viscosa* and some variations in morpho-anatomic features.

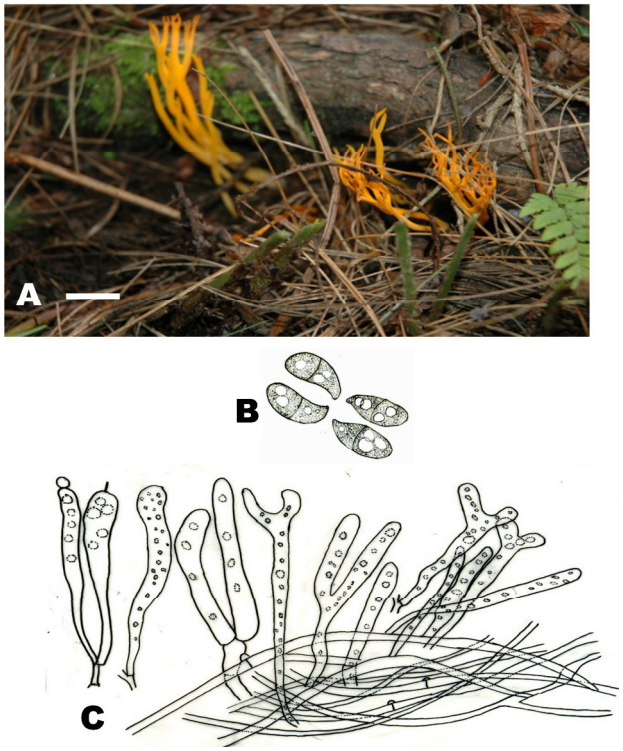

Fig. 1: *Calocera himalayca* sp. nov. Hanif and Nasir: Figure (A) Basidioma (B) Basidiospores (C) Probasidia and tramal hyphae
Scale Bar (1 cm): For **A** 0.5 cm, for **B** 3.2 µm and for **C** 10 µm

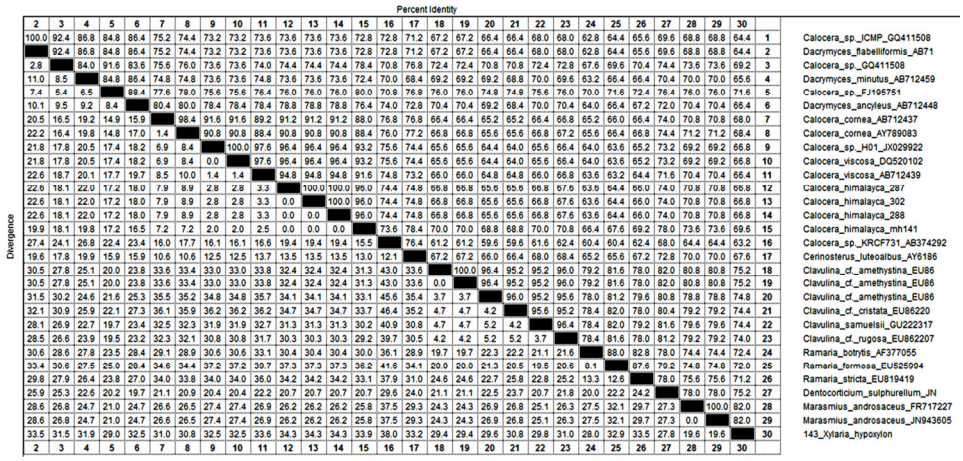

Fig. 2: Percent identities compare sequences directly without accounting for phylogenetic relationships. Percent divergence calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign (DNASTAR)

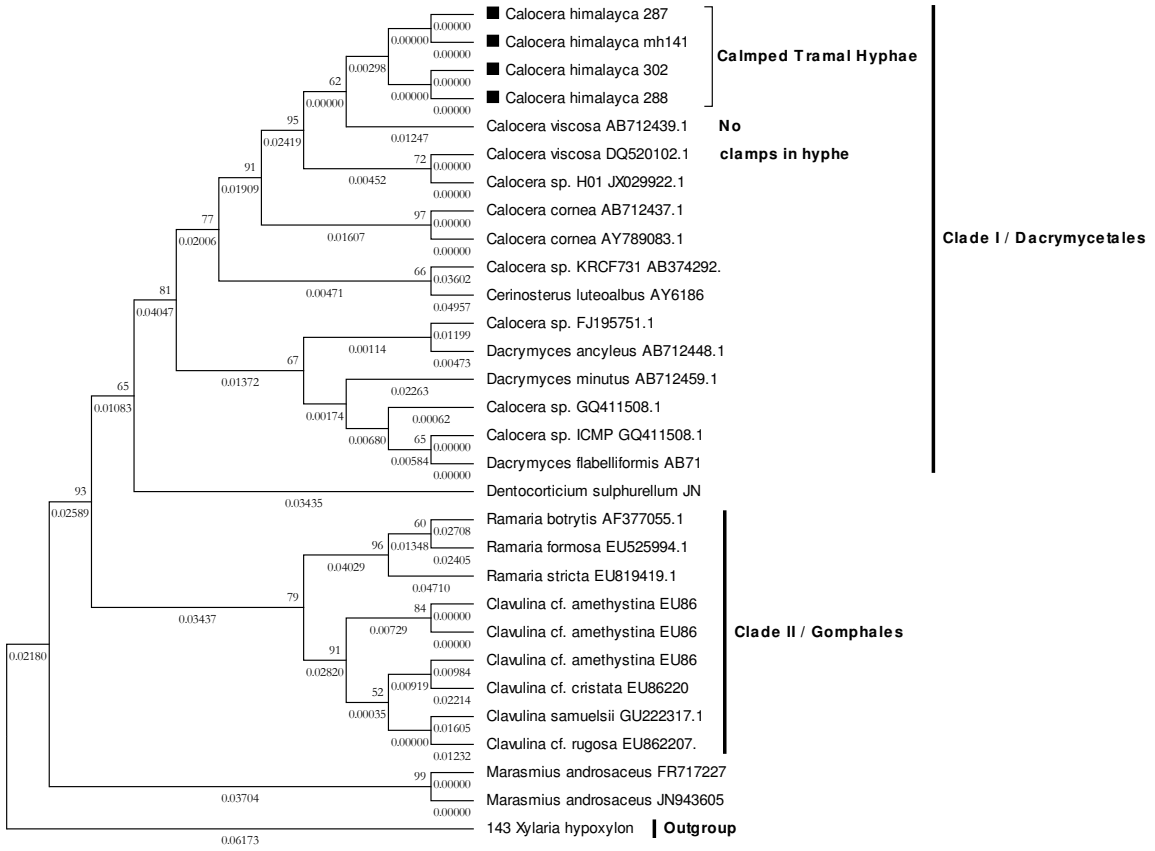

Fig. 3: Evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the 25 taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated

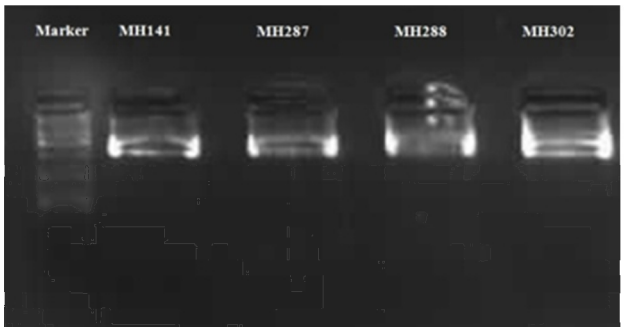

Fig. 4: PCR products of rDNA-ITS regions of *Calocera himalayca* sp. nov. isolates

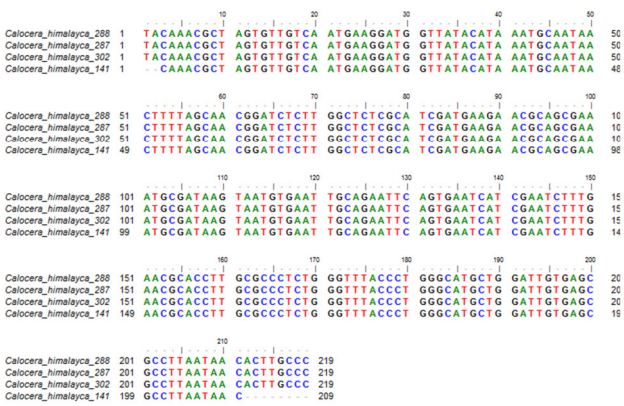

Fig. 5: Alignment of rDNA-ITS sequences of *Calocera himalayca* isolates

C. himalayca was found to grow in a group of 2–4 basidiomata, Murree, Khanspur, Nathiagali, Fairy Meadow, Pakistan. While *C. viscosa* grows scattered on conifer wood. Both *C. himalayca* and *C. viscosa* are similar in colour and size of basidiomata. Spores of *C. viscosa* are longer (7.5–15 µm) than *C. himalayca*. Presence of clamps in tramal hyphae (Fig. 1C) of *C. himalayca* is another unique feature that delimits *C. viscosa* from it. *C. himalayca* also differs from *C. cornea* reported from Pakistan. *C. cornea* has relatively small, simple, slightly branched, palmate or dendroid basidiomata, white to yellow colour, clampless hyphae and slightly longer (7.5–12.5 µm) basidiospores. Phylogenetically, *C. himalayca* clustered within clade I (Decrymcetales) near *C. viscosa* (DQ520102.1) and *C. cornea* (AY89083.1) with strong (91%) bootstrap frequency (Fig. 3). All these three species closely resemble in jelly like appearance of their basidiomata. *C. himalayca* differs from rest of two in having clamped tramal hyphae. These morpho-anatomic differences and interspecific rDNA-ITS variations of *C. himalayca* compared with *C. viscosa* and *C. cornea* are the evidences for novelty of the described species.

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