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New record of *Ascorhizoctonia praecox* (*Tricharina praecox*) from India

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ABSTRACT

***Tricharina*, an interesting genus among the most complex genera of order *Pezizales* in class *Pezizomycetes* is known for its cup-shaped fruiting bodies. However, interspecific distinctions and correct identifications are difficult on the basis of morphological features. So far, single species of the genus *Tricharina* has been recorded from India. However, we recovered *Ascorhizoctonia praecox* (*Tricharina praecox*) while exploring the endophytic mycobiome of *Ephedra gerardiana*. This study highlights the first authentic report of the species being recorded for India. A comprehensive analysis of morphological, molecular, and phylogenetic details is carried out.**

Keywords: *Pezizomycetes*, Endophyte, Phylogeny, Ladakh, New record

INTRODUCTION

Endophytes are the microbes that reside asymptomatically inside the healthy plant tissues without exhibiting visible symptoms of infection (Bacon and White, 2000). Fungal endophytes comprises an essential component of plant endospheric microbiome conferring direct and indirect benefits to the host plants (Afzal *et al.,* 2019). In the recent decade, endophytic fungi have gained immense attention on their diversity, bioprospection, and the multifaceted interactions with their hosts and the related microbiomes of the symbiotic continnum (Alam *et al.,* 2021).

Pezizomycetes comprises a diverse group of fungi exhibiting cosmopolitan distribution with majority being the saprobes, many as mycorrhizal partners, a few parasitic and endophytic to plants. So far, 2000 species belonging to 22 families and one order are recognized in this class worldwide (Pfister and Healy, 2021). The genus *Tricharina* Eckblad (1968: 60), a member of *Pyronemataceae* (*Pezizales*) was proposed to replace an illegitimate name, *Tricharia* Boudier (1885: 104), the latter being the homonym of *Tricharia* Fée (1824: 87). The name *Tricharina* was given to the apothecial teleomorphic stages while anamorphic states were described as *Ascorhizoctonia* (Yang and Korf, 1985a). Later, the genus *Tricharina* was emended and monographed with the inclusion of 12 taxa, while transferring many taxa to the new genus *Wilcoxina*, based on morphological features of apothecia (Yang and Korf, 1985b). Lindemann (2013) tried to clarify some taxonomical aspects and proposed a new key to the 12 accepted taxa. Detailed phylogenetic analyses of the family *Pyronemataceae* revealed paraphyletic nature of *Tricharina* (Van Vooren *et al.,* 2015a, b).

Tricharina praecox (P. Karst.) Dennis was first described in 1971 as a cup-shaped fungal species in the family *Pyronemataceae* (Dennis, 1971). Yang and Korf (1985b) recognized two new varieties to the taxon *T. praecox* var. *intermedia* and *T. praecox* var. *cretea*, while the type species was designated as *T. praecox* var. *praecox*. However, *T. praecox* var. *cretea* was later raised as *T. cretea* (now changed to *T. indica*) and *T. praecox* var. *intermedia* was combined with *T. praecox* var. *praecox* (Van Vooren *et al.,* 2017, 2019). Morphologically, it is difficult to distinguish between the species of this genus, especially between *T. praecox* and *T. gilv*a (Van Vooren *et al.,* 2017). Both these fungi are found in the post-fire environments, however, *T. praecox* is found to be strictly pyrophilous. They are believed to thrive in habitats with pyrolyzed or partially burnt forms of carbon and/or other unique post-fire compounds. Recently, the genus *Tricharina* was emended twice with major modifications. In coherence with the Art. 59 of ICNafp (International Code of Nomenclature for algae, fungi and plants), the species belonging to the "*T. praecox* clade" were reassigned to *Ascorhizoctonia praecox* (Van Vooren *et al.,* 2017). The presence of a number of endophytic sequences in the "*Tricharina* core clade" indicates the possible endophytic lifestyle of the taxon. In the current study, *Tricharina praecox* ES1 was isolated as an endophyte from a gymnosperm, *Ephedra gerardiana* Wall. ex Stapf. Detailed account of isolation, morphological, and molecular characterization with comparison of the data availability is provided.

MATERIALS AND METHODS

Sample collection and isolation of endophytic fungi

Asymptomatic plant samples of *Ephedra gerardiana* were randomly collected in aseptic polythene bags from Namkila Pass (3700 masl), Kargil (Ladakh), India. Root and stem segments were cut into small fragments (5-10 cm) discarding the intermittent segments and washed under tap water to remove the debris followed by a thorough washing with sterile distilled water (SDW). Surface sterilization was done following Petrini (1986) with slight modifications by immersing the samples in 70% ethanol for 3 minutes, 5% NaOCl for 2 minutes, 80% ethanol for 1 minute, and rinsed twice with SDW. The plant fragments were further cut into smaller segments (3-4 mm) using sterile scalpel before placing them aseptically on the potato dextrose agar (PDA) medium contained in 90 mm Petri plates (Borosil®) supplemented with streptomycin (250mg/l). The plates were wrapped with parafilm and incubated at 28±2ºC. After checking the emergence of mycelial threads, the fungal

isolates were grouped as morphospecies based on their similarity. Colonization frequency of each endophytic fungus was calculated as:-

CF % = Total number of isolates of a fungus/Total number of segments inoculated × 100

Sporulation induction and morphological analysis

Endophytic isolates were grown on diverse media, like, oatmeal agar (OMA), corn meal agar (CMA), Spezeiller Nahrstoffarmer Agar (SNA), and water agar (WA) to check the influence of various carbon and nitrogen sources for inducing sporulation.

Fungal isolates were checked for macroscopic/ cultural and microscopic features. Cultural features included color, texture, odour, size, exudates and growth pattern while microscopic features encompassed hyphae colour, texture, septation, fruiting body, spore characters, etc. Micromorphological analysis was accomplished under in-built compound light microscope (Magnus, India) and Digital inverted microscope (Evos® FL Cell Imaging System, Thermo Fischer Scientific, India Pvt. Ltd.) with attached camera using lactophenol cotton blue.

DNA isolation, PCR amplification, sequencing and phylogenetic analysis

Fungal genomic DNA extraction was performed adopting phenol-chloroform method as per the standardized protocol (Sambrook *et al.,* 1989). The partial ITS (Internal Transcribed Spacer) regions from nrDNA were amplified using the universal primers ITS1F [5'-TCC GTA GGT GAACCT GCG G -3'] and ITS4 [5'-TCC TCC GCT TAT TGA TAT GC -3'] (White *et al.,* 1990). The fragment corresponding to ITS1-5.8S-ITS2 nrDNA region was amplified. Thermal cycler was programmed to perform an initial denaturation at 95^{0}C for 4 min, followed by 30 cycles at 95^{0}C for 1 min, 50^{0}C for 20s and 72^{0}C for 1 min, with the extension at 72^{0}C for 15s, followed by final extension at 72^{0}C for 6 minutes. The amplified Polymerase Chain Reaction (PCR) products were purified by PEG-NaCl precipitation. Sequencing was directly carried out on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per the manufacturer's protocol.

The high quality consensus sequence was generated and compared against the top 100 hits submitted in the GenBank database, National Centre for Biotechnology Information (NCBI) site by using the Basic Local Alignment Search Tool (BLASTn) algorithm (www.ncbi.nlm.nih.gov/BLAST). The accession number (MW205788) corresponding to the ITS sequence was procured following its submission in GenBank, NCBI, USA. The closest reference sequences (top hits in GenBank) were selected to reveal the phylogenetic relationships among the taxa under treatment, using *Geopora cercocarpi* and *G. arenicola* as outgroup (**Fig. 1**) Alignment was performed in MAFFT (Multiple Sequence Alignment Tool) version-7 (https://mafft.cbrc.jp). Phylogenetic tree was constructed in Mega XI software (Molecular Evolutionary Genetic Analysis Version XI (Tamura *et al.,* 2021) using maximum likelihood analysis (ML) and Kimura 2 parameter (Tamura *et al.,* 2013), with the testing score of 1000 replicates bootstrap.

RESULTS

Isolation and sporulation induction

Out of 1083 segments, two surface sterilized stem segments of *E. gerardiana* collected in summer season (July) exhibited the emergence of brown mycelia from their cut ends after 7th day of incubation at 28^{0}C (**Fig. 2a**). These two isolates after examining their cultural and micromorphological features were considered as single morphospecies designated as "ES1", showing the colonization frequency (CF %) of 0.18%. The purified colonies of ES1 transferred to various media were frequently examined for their sporulation, however, they failed to sporulate even after incubation period of 3-4 months.

Phylogenetic analysis

The nrDNA ITS1-5.8S-ITS2 sequence of ES1 showed 99.16% similarity with *Tricharina praecox* type strain after BLAST searches in GenBank database (https://www.

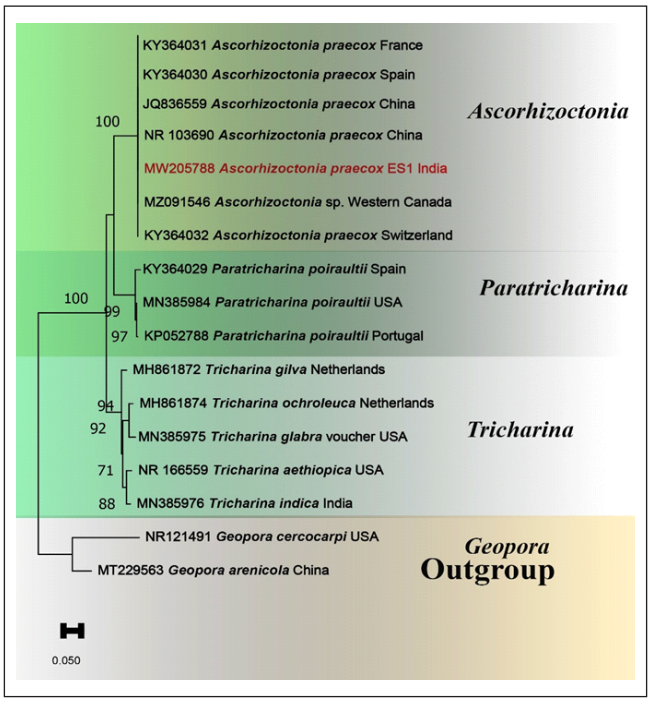

Fig. 1: Phylogenetic relationships of *Ascorhizoctonia praecox* (*Tricharina praecox*) inferred from nrDNA ITS 1-5.8S-ITS 2. Meaningful bootstrap support values (>50%) resulting from maximum likelihood (ML) method are shown on the left of the branches at nodes. *Ascorhizoctonia praecox* is highlighted red in the phylogram.

ncbi.nlm.nih.gov). In a dataset based on 17 nrDNA ITS1-5.8S-ITS2 sequences (alongwith our sequence) retrieved from authentic published phylogenies, encompassing four genera (*Ascorhizoctonia, Paratricharina, Tricharina,* and *Geopora*) of the family *Pyronemataceae*, ES1 strongly clustered within the clade of *Ascorhizoctonia praecox* with the support value of 100% bootstrap (**Fig. 1**).

Taxonomy

T*richarina praecox* (P. Karst.) Dennis (*Pyronemataceae*), Kew Bulletin **25(2):** 338. 1971 (**Fig. 2 & 3**).

Heterotypic synonym and new name: *Ascorhizoctonia praecox* Chin S. Yang & Korf (*Pyronemataceae*), Mycotaxon **23:** 475, 1985.

Description: Colonies immersed, closely adhered to the medium, shiny brown, extremely slow-growing, with spreading floccose off-white mycelial threads arising from the periphery, odour inconspicuous.

On PDA, colonies showed restricted growth, attained 31-37 mm diameter after 21 days of incubation at 28ºC, greyish in the centre, surrounded by a narrow dark-brown ring, followed by broad light-brown concentric ring with ill-demarcated margins (**Fig. 2b**), peripheral off-white mycelial hairs floccose, spreading outward, fast-growing, emerging approximately after 11 days of incubation; reverse brown. On MEA, colonies closely appressed to medium, 28-32 mm in diameter after 21 days at 28ºC, shiny brown, slightly more shiner than that on PDA, crystal-like exudates oozing out of the central greyish region, peripheral hair growing towards the upper plate, growth slightly lesser than that on PDA (**Fig. 2c**); reverse dull brown with elevated off-white peripheral hairs. On CDA, colonies completely appressed to the medium, 15-18 mm in diameter after 21 days at 28ºC, dull brown, greyish centre not demarcated, concentric rings absent, margins diffuse, peripheral hairs absent (**Fig. 2d**); reverse light brown.

Mycelium light brown to dark brown, consisting of septate, branched, smooth as well as rough walled hyphae (**Fig. 3a**). Smooth-walled hyphae further comprised of two types of cells - narrow, elongated, rectangular cells, roughly thrice in length than breadth, light to golden-brown (**Fig. 3b**), (4.8) 8-14.4 (16.1) µm wide; certain cells extrude a peculiar outgrowth through the longer wall which gradually inflates and divides to give rise to highly inflated, branched, (19.2) 21.7-30.5 (34.0) broad hyphae (**Fig. 3c, d**). Rough-walled hyphae comprised of long, rectangular and echinulated cells, (6.1) 8-11 (13.4) µm wide, light to dark brown, with black ornamentally designed wall, bearing minute denticles (**Fig. 3e**).

Habit and Habitat: Endophytic to the stem of *Ephedra gerardiana* growing in a cold arid desert with high UV radiations and water scarcity.

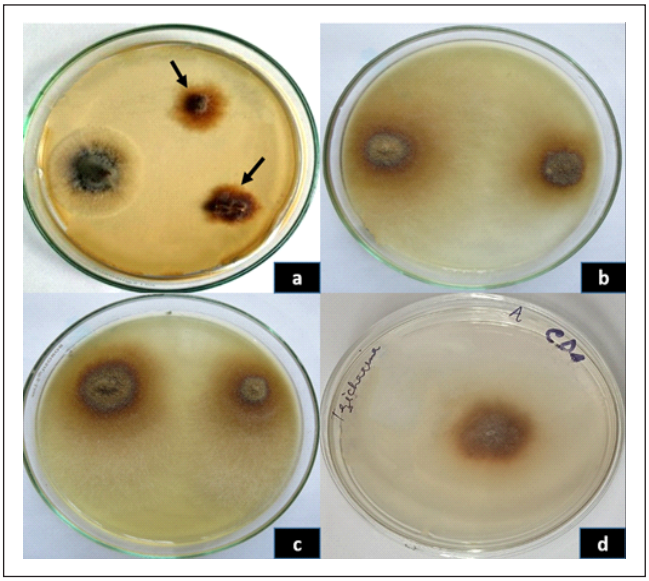

Fig. 2: Cultural characteristics of *Ascorhizoctonia praecox* a) Isolation plate showing emergence of brown mycelia from two stem segments; Colonies on b) PDA, c) MEA, d) CDA

Distribution: USA, United Kingdom of Great Britain and Northern Ireland, Spain, Russian Federation, China, Canada, Luxembourg (Yang and Korf, 1985; Hansen and Knudsen, 2000; GBIF, 2020).

Material examined: India, Ladakh, district Kargil, Namkila Pass, 34º20′.10″N, 76º33′.21″E, 3691 m.a.s.l., 17 July, 2019, Aroosa Jan Mattoo and Skarma Nonzom (ES1).

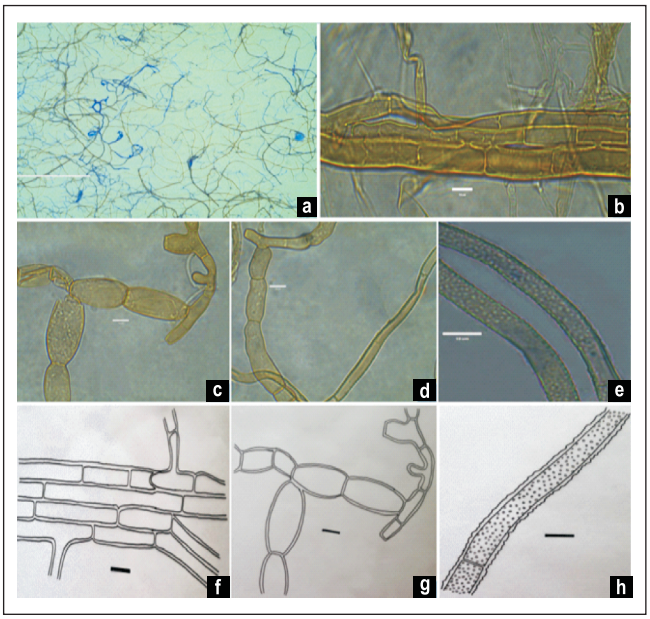

Fig. 3: *Ascorhizoctonia praecox* a. Immature mycelium b. Part of mature mycelium showing narrow smooth-walled hyphae c-d. Extrusion of broad-celled, smooth-walled hyphae e. A portion of echinulated hyphae f-h. Line drawings of narrow smooth-walled (f), broad smooth-walled (g), and echinulated hyphae (h). Scale bars: a = 200 µm; b-h = 10 µm

DISCUSSION AND CONCLUSION

Tricharina praecox is a rare taxon with only 16, 18, and 1 records in NCBI GenBank (https://www.ncbi.nlm.nih.gov), Global Biodiversity Internet Facilities (GBIF 2020), and U.S. National Fungal Collection (https://nt.ars-grin.gov), respectively, while four records each in Mycobank (http://www.mycobank.org/) and Index fungorum (www.indexfungorum.org). So far, the species has been found endophytic to *Juniperus* (Hoffman and Arnold, 2010), *Salix sitchensis*, and *Pseudosuga menziensis* (Wolfe *et al.,* 2022). Also, to the best of our knowledge, reports regarding association of *T. praecox* with *E. gerardiana* are lacking, therefore, this study showcases the pioneer host record of the concerned species.

Although data on cultural characteristics was not available for comparison, phylogenetic analysis showed strong clustering of ES1 in the *Ascorhizoctonia praecox* group with the bootstrap value of 100%, in between its counterparts from China and Canada (**Fig. 1**). Based on cultural characteristics, this species can be identified as extremely slow growing, immersed, and shiny brown colony. Molecular characterization based on the primary barcode markers (ITS1 & ITS2) has proved to be a better tool for identification as morphology poses certain confusions while demarcating different species in *Tricharina*. Keeping in view the rare distribution of this taxon, its recovery from a plant inhabiting an oligotrophic region with high incidence of UV radiations might be correlated to it's predominantly pyrophilous nature.

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