Bangladesh J. Plant Taxon. **29**(1): 129-136, 2022 (June) DOI: https://doi.org/10.3329/bjpt.v29i1.60453

FIRST REPORT OF THE ECTOMYCORRHIZAL STATUS OF *CLAVARIADELPHUS PAKISTANICUS* HANIF & KHALID BASED ON MORPHOTYPING AND MOLECULAR EVIDENCE

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Keywords: Aphyllophorales; Basidiomycetes; Khanspur; Morphotypes; nrDNA; Symbiosis.

Abstract

The ectomycorrhizae of a newly described club fungus *Clavariadelphus pakistanicus* Hanif & Khalid were collected from Ayubia, Khyber Pakhtunkhwa, Pakistan and described morpho-anatomically. Its *Pinus wallichiana* associated ectomycorrhizae have been characterized by dichotomously branched, reddish brown color of mature and dark brown to blackish young ectomycorrhizal tips with frequent unbranched and septate emanating hyphae. During molecular and phylogenetic analyses, these mycobionts showed maximum similarity and were clustered with basidiocarps sequences of *C. pakistanicus*. Hence it was confirmed that these ectomycorrhizae belong to *C. pakistanicus* and being first time reported from Pakistan.

Introduction

Clavariadelphus Donk is widely distributed in the temperate forests and has 20 species worldwide (Methven, 1990; Kirk *et al*., 2008; Hanif *et al*., 2014). *Clavariadelphus* is generally considered to be an element in the biota of the Northern Coniferous Forest and several species in North America are confined to mixed deciduous coniferous forests (Methven, 1990). Little is known about the nutritional status of the *Clavariadelphus* species.

Pines are generally considered as ideal hosts for many of the ectomycorrhizal (ECM) fungi (Hanif *et al.*, 2012; Hanif, 2012). There are many reports that indicate the ectomycorrhizal nature of pines. Some examples from literature are: Visser (1995) determined *Coltricia perrinis*, *Thelephora* spp., *Suillus brevipes*, *Cenococcum geophillum*, *Cortinarius* spp., *Lactarius* spp., *Russula* spp., *Tricholoma* spp. as ECM fungi with *Pinus banksiana*. Guo *et al.* (2020) reported 104 ectomycorrhizal operational taxonomic units (OTUs) from *Pinus sylvestris* roots. Zhao *et al.* (2020) claimed to isolate 805 OTUs from the same host. Hilszczańska *et al.* (2011) reported ectomycorrhizal symbiosis in *P. sylvestris* with *Suillus luteus*, *Thelophora terrestris*, *Tomentella* spp., *Dermocybe palustris* and *Dermocybe* spp. Margit *et al.* (2010) reported the different mycobionts (*Amphinema byssoides*, *Wilcoxina* sp. *Flexipes*, *Suillus ploranus* and *Tomentella*) in different host plants including *P. cembra.* Hawley *et al.* (2008) reported the ECM of *Phialocephala tortnii* and *Hymenocyphus ericae* with *Pinus patula.* Chung *et al.* (2003) reported many ectomycorrhizal fungi with *P. densiflora* and *P. rigita*. Koizumi and Nara (2019) reported 154 ECM fungal species from the root tips of *P. pumila*. Niazi *et al.* (2010) reported the ECM of *Cantharellus cibarius* with *P. wallichiana* from the Himalayan Temperate Forest of Pakistan. Tyub *et al.* (2018) reported 33 fungal taxa associated with *P. wallichiana* out of which 23 were ectomycorrhizal and rest were non-mycorrhizal. Murata *et al.* (2017) reported 42 putative ECM

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fungi in association with *P. amamiana*. *Clavariadelphus* species are reported as mycorrhizal with diverse hosts *i.e*., *Clavariadelphus americanus* is reported as mycorrhizal with oaks and pines (Corner, 1950; Methven, 1989, 1990; Kuo, 2007), *C. occidentalis* with conifers (Methven, 1989).

Mostly ectomycorrhizal mushrooms including *Clavariadelphus* are widely distributed in the moist temperate forests of the World. Pakistan also has diversity rich hotspot areas in Himalayan moist temperate region and many mushrooms along with their mycobionts have been documented from this region (Niazi, 2008; Hanif, 2012; Sarwar, 2012; Ilyas, 2013; Jabeen, 2016) but unfortunately very little is known about complete picture of diversity of these fungi. Previously, Niazi (2008) described the ECM of *Clavariadelphus truncatus* with *P. wallichiana*. Some club fungi fruiting bodies have been reported by Hanif (2012) but their ectomycorrhizae are not well reported from Pakistan. In the present investigation, fruiting body of the *C. pakistanicus* and its ECM is illustrated and described morpho-anatomically and phylogenetically from the roots *P. wallichiana*. It is the first report of the mycorrhizal status of *C. pakistanicus* from Pakistan.

Materials and Methods

Isolation and clearing of ectomycorrhizae

The sampling was carried out during the rainy season (July–August) from the coniferous forests of Pakistan located at an elevation of around 2200 m.a.s.l. Ectomycorrhizae of *Clavariadelphus pakistanicus* associated with *Pinus wallichiana* were sampled by tracing the rhizomorphs extending from the base of fruiting bodies towards plant roots. Soil blocks with roots were dug and packed in polythene bags and brought in laboratory for further analyses. The sampled roots were cleaned with running tap water and mycorrhizal roots were separated from non-mycorrhizal roots. The unramified ends of ectomycorrhizal morphotypes were cut in such a way to retain the particular system. These were then preserved in 2% CTAB buffer. The ectomycorrhizal systems were studied morpho-anatomically with the help of stereo microscope (for morphological studies) and compound microscope (for anatomical studies).

Morphological studies

The ectomycorrhizal system was studied under stereo microscope for length of mycorrhizal system, length of unramified ends, diameter of unramified ends, diameter of axis, branching system, shape of unramified ends, distinct features of mantle surface and the color of system following Agerer (1991, 1987–2002)

Anatomical description

Mantle was peeled off under stereo microscope in one drop of lactic acid and observed under compound microscope to study anatomical features of mantle surface like hyphal arrangement, shape and size of cells and dimensions of hyphal cells and drawn with Camera Lucida.

Molecular characterization and phylogenetic analysis

DNA was extracted from ECM root tips by following a modified CTAB method (Gardes and Bruns, 1996). Primer pairs ITS1F/ITS4 (White *et al*., 1990) for the ITS region were used for PCR and Sanger sequencing. All PCR products were evaluated for successful amplification using SYBR Green and 1.5% agarose gels with TAE buffer for gel electrophoresis. Amplicons were prepared for sequencing *via* enzymatic purification using exonuclease I and shrimp alkaline phosphatase enzymes (Werle *et al*., 1994). Purified products were sequenced through MacroGen Company (Seoul, South Korea). Sequence chromatograms were trimmed, edited, and assembled using Sequencher4.1 (Gene Codes, Ann Arbor, MI). Consensus sequences were analyzed using BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/). The most similar sequences for ITS

region were retrieved from GenBank. These ITS sequences were then aligned using MUSCLE Alignment Tool to generate alignments (Edgar, 2004). MEGA5 software was used for phylogenetic analysis with maximum likelihood criterion by following algorithm and Jukes and Cantor (1969) model of sequences evolution (Tamura *et al.*, 2011). One thousand bootstrap iterations were performed with rapid bootstrapping significant support was considered to be ≥70%. All phylogenetic analyses were performed on the CIPRES Portal v. 3.1. (Miller *et al.*, 2010).

Results and Discussion

Morpho-anatomical characterization of ectomycorrhizal system of Clavariadelphus pakistanicus

Ectomycorrhizal system dichotomous, main axis 4–4.5mm long, axis 0.5mm in diameter. unramified ends bent, 1mm long and 0.5mm in diameter, younger unramified ends reddish brown, older ends blackish brown. Texture of the system was smooth, host tissues not visible under the sheath; mantle surface smooth or cottony; Rhizomorphs absent. Emanating hyphae were common, concentrated around the sides of unramified ends, honey brown in colour (Fig. 1A-D).

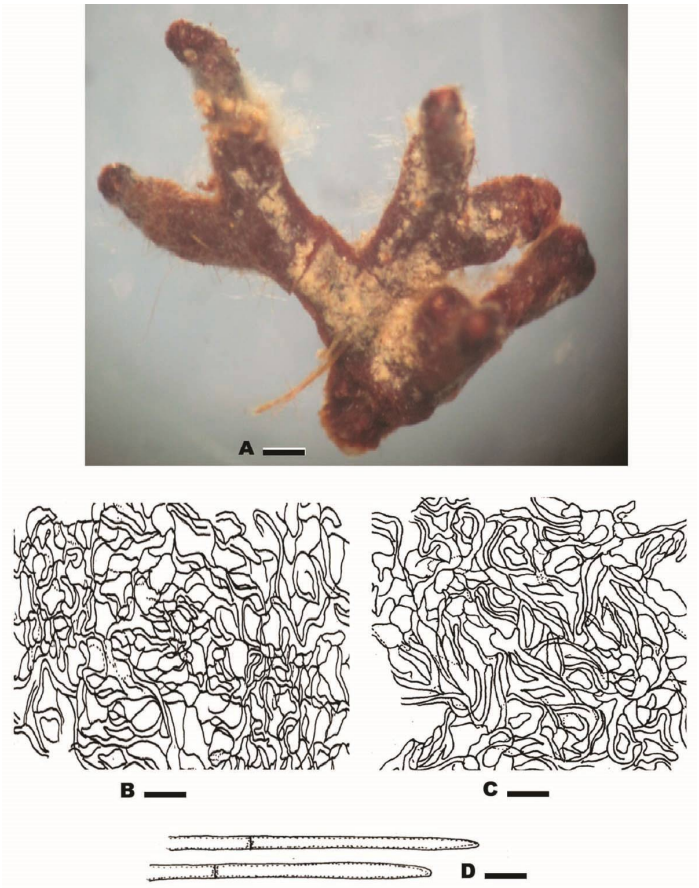

Fig. 1. Ectomycorrhizae of *Clavariadelphus pakistanicus*, A. ECM (habit) showing important morphological features; B. Pseudoparenchymatous (type M) outer mantle; C. Pseudoparenchymatous (type M) inner mantle; D. Emanating hyphae. Scale Bar: for A= 0.7cm; B = 0.41µm; C = 0.52 µm; D = 0.29 µm.

Outer mantle layer pseudoparanchymatous, cells 6.24 µm in length and 1.35µm wide, matrix material pale yellow, densely packed round lobed cells, epidermoid cells of pale yellow colour, hyphae compactly packed and forked. Inner mantle layer also pseudoparanchymatous, gelatinous matrix material visible, cells 7.9µm long and 1.2µm wide, cells were same in size as the outer mantle, cells contents not clear (Fig. 1 (A-D). Rhizomorphs absent, emanating hyphae common, clamps absent, septate, unbranched, cylindrical hyphae, not constricted at the septa, cell content clear, cells ~0.58µm width and 38.6µm long, cells thick walled.

Molecular identification and Phylogenetic analysis

The morpho-anatomic identification of ectomycorrhizae was supported by rDNA-ITS sequence based molecular identification. Sequences originated from the ITS region were used as a reference to BLAST against GenBank data. All sequences showed maximum similarity (100%) with *Clavariadelphus pakistanicus* sequences of sporocarp (HQ379937). Similar sequences were retrieved from GenBank and aligned with Pakistani ectomycorrhizal sequences reported during this study. Final data set for phylogenetic tree included 20 sequences. Tree was constructed through maximum likelihood criterion and showed highest log likelihood (-2451.4566). Phylogram consisted of 2 major clades and a few independent leaves (Fig. 2). Ectomycorrhizae of *C. pakistanicus* clustered with its basidioma (mh99, mh126, mh129), the above ground partner with strong bootstrap percentage (99%). All sequences of *C. pakistanicus* nested within clade of species that were previously reported as ectomycorrhizal with various photobionts. Placement of all these species with *C. pakistanicus* indicates ectomycorrhizal status.

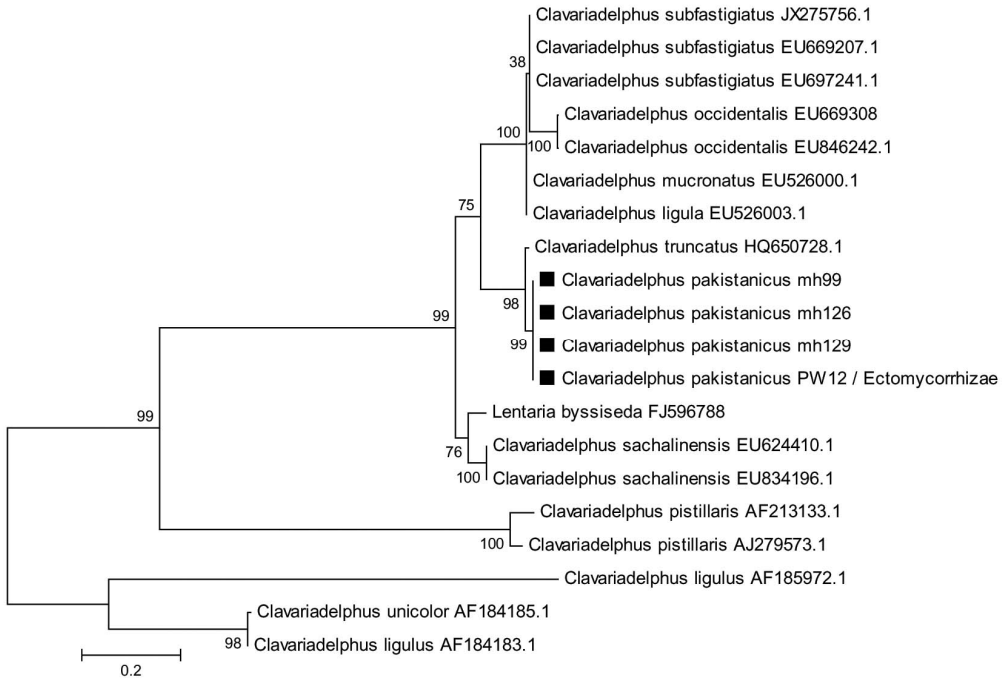

Fig. 2. Phylogenetic position of of *Clavariadelphus pakistanicus* ectomycorrhizae from Pakistan with respect to other related spp. Tree inferred by maximum likelihood analysis based on rDNA sequences, including ITS region. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession numbers are given at the end of species names.■ indicate species reported from Pakistan.

Interaction between photobionts and mycobionts is the archetype of symbiosis or mutualism (Ågren *et al.*, 2019). These mycorrhizal associations are beneficial to the plant. The identification of ectomycorrhizal morphotypes based on morphological criteria is difficult to disseminate species. Therefore, the use of molecular techniques is an effective alternative. During present work ectomycorrhizal morphotype of a novel club fungus *Clavariadelphus pakistanicus* (Hanif *et al*., 2014) has been reported from Pakistan growing with *Pinus wallichiana*. This is the first report of ectomycorrhizae of this fungus from the World. Previously ectomycorrhizal association of *Clavariadelphus ligula* (Quel.) Donk was found at Hurpora and Yusmarg with *Cedrus deodara* and *Pinus wallichiana* (Itoo and Reshi, 2014). Ectomycorrhizal morphotypes of *C. pakistanicus* were collected by tracing method and identified through its rDNA-ITS sequence following Landeweert *et al.* (2003) and characterized by morphotyping method (Agerer, 1991; Agerer, 1987–2002; Mello *et al*., 2006). Clasen *et al.* (2018) reported that molecular tools based on sequencing of rDNA-ITS could be effective in species characterization and phylogenetic analysis. Their ectomycorrhizae have dichotomous ectomycorrhizal systems with bent unramified ends (reddish brown when young and blackish brown when old). Literature showed that mostly *Pinus* associated ectomycorrhizae have dichotomous branching pattern (Agerer, 1987–2002). Rhizomorphs were not recorded although some rhizomorphs like structures were present but their morphology and anatomy not support them as rhizomorphs. Emanating hyphae were frequent. Mantle organization in both outer and inner view was pseudoparanchymatous. These basic structure features may resemble with *Pinirhiza lactariosimilis* associated *Pinus sylvestris* (Golldack *et al*., 1997) but *P. lactariosimilis* may have some structural differences as well. ECM of *C. pakistanicus* has large (4-4.5mm) ectomycorrhizal system than *P. lactariosimilis* (2.7mm). Both these morphotypes also differed in cell size, smaller in later (2.5µm) and larger in earlier (6.24µm). The ECM of *C. pakistanicus* was also compared with the ECM of other related species. Ectomycorrhizae of both *C. trancatus* (Niazi *et al.*, 2010) and *C. pakistanicus* have dichotomous branching. The ramification pattern in *C. pistillaris* was reported as monopodial pinnate associated with *Fagus sylvatica* (Iosifidou and Raidl, 2006). Rhizomorphs absent in *C. pakistanicus* whereas present in *C. trancatus* and *C. pistillaris* (Iosifidou and Raidl, 2006; Niazi, 2008; Niazi *et al.*, 2010). All these three species have emanating hyphae. Mantle organization was pelectenchymatous in *C. trancatus* (Niazi *et al*., 2010). There are very few reports about mycorrhizal status of the species in genus *Clavariadelphus*. *C. americanus* was reported to form ECM with oaks and pines (Corner, 1950; Methven, 1989, 1990), *C. occidentalis* and *C. unicolor* with pines (Corner, 1950; Smith *et al.*, 1981; Methven, 1989, 1990). The ectomycorrhizae of genus *Clavariadelphus* were also reported with *Quercus* spp. and many other deciduous trees (Izzo *et al.*, 2005; Iosifidou and Raidl, 2006; Smith *et al.*, 2007; Morris *et al.*, 2008). *C. mucorantus* and *C. ligula* were reported in association with *Pseudotsuga menziesii* (Smith *et al.*, 2002). The ectomycorrhizae of this fungus may increase the nutrients supply to the host. Same is reported by Corrales *et al.* (2018). They suggested that soils with poor nutrients are abundantly dominated by ectomycorrhizal fungi that can optimize plant nutrition and may contribute to the maintenance of forests. Many different mycobionts are being reported with deciduous hosts (Aryal *et al.*, 2020; Defrenne *et al*., 2019; Albuquerque-Martins *et al*., 2019). Present report is another addition to ectomycorrhizal mycobiont (*C. pakistanicus*) from this genus which in future could help in silviculture practices in forest decline countries like Pakistan.

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(Manuscript received on 01 January, 2021; revised on 03 December, 2021)