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ECTOMYCORRHIZAE OF HIMALAYAN ALDER (*ALNUS NITIDA*) FROM THE MOIST TEMPERATE FORESTS OF PAKISTAN

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Abstract

The roots of the Himalayan alder were sampled from the rhizosphere and were investigated for ectomycorrhizal association. We described 11 ectomycorrhizal species viz; *Amphinema* sp. MHBYS-01, *Helvella* sp. MHELA-02, *Hydnobolites* sp. MHCER-02, *Inocybe* sp. MHNIT-01, *Inocybe* sp. MHNIT-02, *Inocybe* sp. MHOBL-01, *Peziza michelii*, *Sebacina* sp. MHAFE-01, *Tomentella* sp. MHSTU-01, *Tomentella* sp. MHSUB-01 and *Tylospora* sp. MHTYL-01 based on molecular phylogeny by using the Maximum Likelihood criterion. Some of the ectomycorrhizal morphotypes are also being described here on a morpho-anatomical basis.

Key words: Clade, Dichotomous, Khanspur, Pseudoparenchymatous, rDNA-ITS

Introduction

Alnus Mill. comprises approximately 28-44 species and are widely distributed in the boreal and temperate zone of the Northern hemisphere (Rochet *et al.*, 2011). From Pakistan, only the *Alnus nitida* is reported from the Himalayan forests of Pakistan. It is native to the Himalayas and west Nepal.

A. nitida has many uses; decoction of its bark is used for the treatment of swellings and body pains (Gamble, 1972), tannin from the bark is used in dyeing. Its wood is used for construction, timber, firewood, as street tree and furniture (Gupta, 1945; Gamble, 1972; Khan *et al*., 2020). *A. nitida has been* used as fodder (Rana *et al*., 2019), medicinal, construction, furniture, fencing, roofing, utensils (Ahmad *et al*., 2009), *A. nepalensis* used to treat Urination with bleeding in livestock and its extract is given orally (Nand and Naithani, 2018) and *A. nitida* has also been used as a pain reliever and has pain reducing the potency of catkin and cone extracts (Nagina & Ibrar, 2018).

Ectomycorrhizal status of the *Alnus* has been previously proved by a number of studies (Trappe 1964; Molina 1979; Miller *et al.*, 1991, 1992; Pritsch 1996, Pritsch *et al.*, 2000; Becerra *et al.*, 2005; Tedersoo *et al.*, 2009; Ostonen *et al.*, 2009; Kennedy and Hill 2010; McBurney *et al*., 2017; Thiem *et al*., 2018; Kilpeläinen *et al*., 2019).

Alder (*Alnus*) belongs to the birch family Betulaceae. This genus is distributed throughout the north temperate zone. From Pakistan, only *Alnus nitida* (Himalayan alder) is found along the western Himalayas. This host plant remained unexplored for their belowground ectomycorrhizal partners from this region. There was only one report about the ectomycorrhizal evidence of *Alnus nitida* from Pakistan (Ashraf *et al.*, 2012). They described and illustrated ectomycorrhizae of *Peziza michelli.* In the present work additional 11 ectomycorrhizal morphotypes have been identified, described and illustrated with this photobiont.

Material and Methods

Sampling of ectomycorrhizae: The Himalayan alder (*Alnus nitida*) roots were sampled from Ayubia National Park, KPK. Ectomycorrhizal roots were collected, vouchered and wrapped in polythene bags. The morphotypes of *A. nitida* were manually sorted after removing the soil particles from the surface of the morphotypes and kept in 2% CTAB buffer for DNA extraction and in distilled autoclaved H_2O for morpho-anatomic characterization. The selected morphotypes were characterized morpho-anatomically following Agerer (2002). Morphological and anatomical characterization of the ectomycorrhizal system was carried out under stereo and compound microscopes, photographed and illustrations were made with the help of Camera Lucida.

Molecular characterization: For molecular characterization, DNA was extracted from the selected morphotypes using the modified CTAB method given by Gardes & Bruns (1993). Amplification of the extracted DNA was performed using fungal-specific and universal primers (ITS1F, ITS1 and ITS4). Hot-start enzyme JumpStart (Sigma, St Louis, MO, USA) was used to catalyze 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. The DNA sequences were submitted to BLAST and used to query the nucleotide collection using default settings. The divergence in rDNA-ITS was measured by comparing sequence pairs reconstructed by using MegAlign (DNASTAR). DNA sequences obtained from *A. nitida* morphotypes were submitted in GenBank. These sequences were manually edited using MacClade 4.08 and Bioedit (version 7.0.9).

Results

Amphinema sp. MHBYS-01 Plate 01 (Figs 1. A-E)

Morphological characters: ECTOMYCORRHIZAL SYSTEM simple to irregular, axis 30×50-1.0×1.0mm, UNRAMIFIED ENDS dichotomously branched, club-shaped, 3.0×5.0-1.0×1.0mm, young tips skin colored

while oxblood red at maturity. Texture smooth with a matte luster, no visibility of host tissue beneath the mantle sheath. EMANATING HYPHAE frequent, off-white in color, RHIZOMORPHS not observed.

Anatomical characters of mantle: MANTLE transitionally pseudoparenchymatous, OUTER MANTLE plectenchymatous, with irregularly arranged and densely packed hyphae (mantle type B; Agerer, 2006), up to 11.0-11.7µm in diameter, contents clear, pale yellow in color, septa and clamps absent, hyphal junctions absent, matrix material not visible. INNER MANTLE transitionally parenchymatous, hyphae intermingled with epidermoid cells, 8.0-14.0µm in length while 8.0-9.0µm in width, light brown in color, contents clear, ornamentation absent, matrix material not visible.

Anatomical characters of emanating elements: EMANATING HYPHAE frequent, transparent, straight, up to 2.1-2.4µm in diameter, contents clear, ornamentation absent, frequently septate, clamped, branching not observed.

***Helvella* sp. MHELA-02 Plate 01 (Figs. 2A-F)**

Morphological characters: ECTOMYCORRHIZAL SYSTEM simple to irregularly dichotomous, main axis 7.0×10-0.5×0.5mm, UNRAMIFIED ENDS bulbous to club-shaped, 2.5×3.0-1.0×1.0mm, tips light brown to beige in color. Texture of system smooth with a matte luster, host tissue not visible beneath the mantle sheath. Main axis of the Ectomycorrhizal system covered in a dense matte of short pointed echine like structures. EMANATING HYPHAE frequent, concentrated around the tips, white in color. RHIZOMORPHS not observed.

Anatomical characters of mantle: MANTLE pseudoparenchymatous, OUTER MANTLE pseudoparenchymatous with densely packed irregular to rectangular cells (mantle type K; Agerer, 2006), cells 4.0×4.7 µm in width while 6.1×6.8µm in length. Cells pale yellow, contents clear, no matrix material, ornamentation absent. INNER MANTLE pseudoparenchymatous (type K; Agerer, 2006), cells irregular to angular, 3.8×4.6µm in width while 5.9×7.1µm in length, contents clear, no matrix material.

Anatomical characters of emanating hyphae and Echines: EMANATING HYPHAE frequent, straight, hyaline, up to 1.8-2.1µm in diameter, thin walled, moderately septate, clampless, contents clear, ornamentation absent. ECHINE LIKE STRUCTURES on the main axis, 47.2- 94.4µm in length, apex 2.5-2.6µm, mid 3.3-3.5µm and base 5.4-5.6µm in width, contents clear, encrusted with gelatinous material.

***Inocybe* sp. MHNIT-01 Plate 01 (Figs. 3A-F)**

Morphological characters: ECTOMYCORRHIZAL SYSTEM simple to irregular, axis 10.0×15.0-0.5×1.0mm, UNRAMIFIED ENDS simple to irregular, 5.0×7.0-0.5×1.0mm, color of system is rusty-brown; texture of system is coarsely grainy with a matte luster. No visibility of host tissue beneath the mantle sheath. EMANATING HYPHAE frequent, light brown, RHIZOMORPHS not observed.

Anatomical characters of mantle: MANTLE pseudoparenchymatous, OUETR MANTLE pseudoparenchymatous, with tightly packed angular cells (mantle type: K; Agerer, 2006), upto 7.9-8.9µm in length and 8.0-11.4µm in width, pale yellow in color, contents clear, matrix material absent. INNER MANTLE pseudoparenchymatous, densely packed angular cells, pale yellow to hyaline in color, contents clear, matrix material not observed.

Anatomical characters of emanating elements: EMANATING HYPHAE frequent**,** of two distinct types.

Type 1 is straight, dark brown in color, up to 2.1-3.2µm in width, thin-walled, rarely septate, clamps present, ramification present; contents clear except a few oil globules present, ornamentation absent.

Type 2 is straight to wavy, hyaline, up to 10.0-11.1µm in width, thin-walled, rarely septate, clamps absent, contents clear, thickly ornamented with echines, ramification present.

***Sebacina* SP. MHAFE-01 Plate 01 (Figs. 4A-F)**

Morphological characters: ECTOMYCORRHIZAL SYSTEM simple to irregular, axis 25.0×35.0-0.5×1.0mm, UNRAMIFIED ENDS simple to bulbous, 3.0×7.0-1.0×1.0mm, young tips light skin to beige in color while dark at maturity. Texture of system smooth with a matte luster. Host tissue not visible beneath the mantle sheath. EMANATING HYPHAE present, RHIZOMPORPS not observed.

Anatomical characters of mantle: MANTLE plectenchymatous, OUETR MANTLE plectenchymatous with irregularly arranged hyphae (mantle type B; Agerer, 2006), up to 3.1-5.1µm in width, hyaline, some hyphae terminally branched, contents clear, septa present, clamps absent, hyphal junctions absent, matrix material not visible. INNER MANTLE plectenchymatous, hyphae up to 3.2-4.7µm in width, contents clear, hyaline, septate, clamps absent, hyphal junctions absent, matrix material not visible.

Anatomical characters of emanating elements: EMANATING HYPHAE frequent, densely surrounding the ectomycorrhizal system. Two distinct types of emanating hyphae present.

Type 1 light brown, up to 5.7- 6.1µm in width, straight to wavy, thick-walled, rarely septate, unbranched, encrusted with gelatinous material, contents clear.

Type 2 hyaline, up to 1.8-2.1µm in width, straight, thin-walled, moderately septate, clamps present, ornamentation absent, contents clear.

***Tomentella* sp. MHSTU-01 Plate 01 (Figs. 5A-F)**

Morphological characters: ECTOMYCORRHIZAL SYSTEM simple to irregular, axis 7.0×11.0-0.5×1.0mm, UNRAMIFIED ENDS simple, 2.0×4.0-0.5×1.0mm, tips black in color, texture of system coarse with a matte luster. Host tissue not visible beneath the mantle sheath. EMANATING HYPHAE present, concentrated on tips. RHIZOMORPHS present.

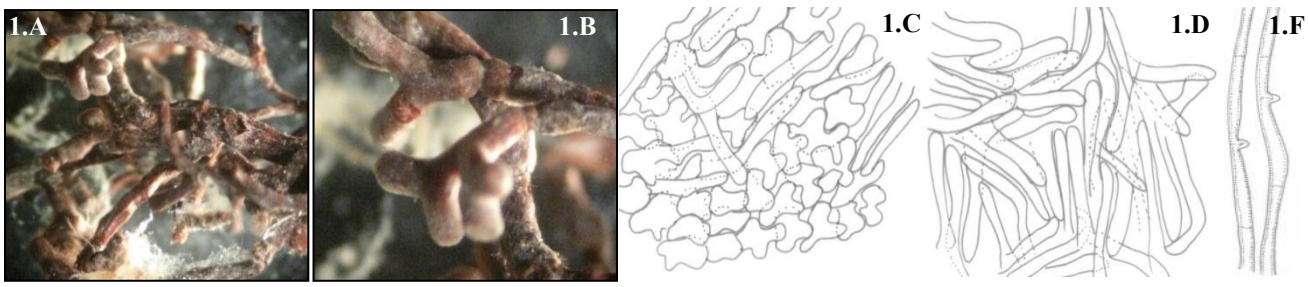

Fig. 1. A-E. Morpho-anatomical features of *Amphinema* sp. MHBYS-01 (A-B) ECM morphotypes of *Amphinema* sp. MHBYS-01 (C) Transitionally pseudoparenchymatous outer mantle (D) Plectenchymatous Inner Mantle (E) Emanating Hypahe.

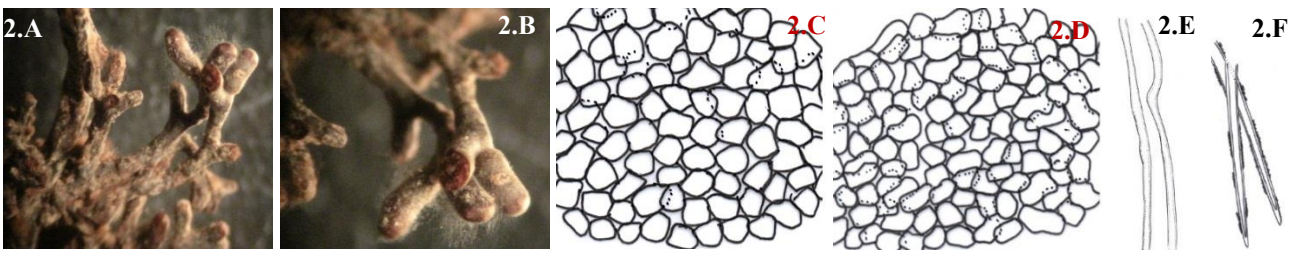

Fig. 2. A-F. Morpho-anatomical features of *Helvella elastica* (A-B) ECM morphotypes of *Helvella elastica* (C) Pseudoparenchymatous outer mantle (D) Pseudoparenchymatous Inner mantle (E) Emanating Hyphae (F) Scale-like Hairs.

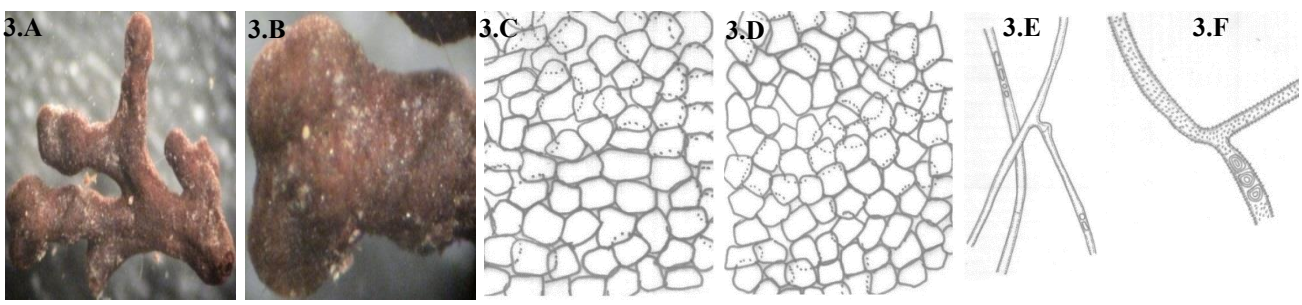

Fig. 3. A-F: Morpho-anatomical features of *Inocybe nitidiscula* (A-B) ECM morphotypes of *Inocybe nitidiscula* (C) Pseudoparenchymatous outer mantle (D) Pseudoparenchymatous inner mantle (E-F) Emanating Hyphae.

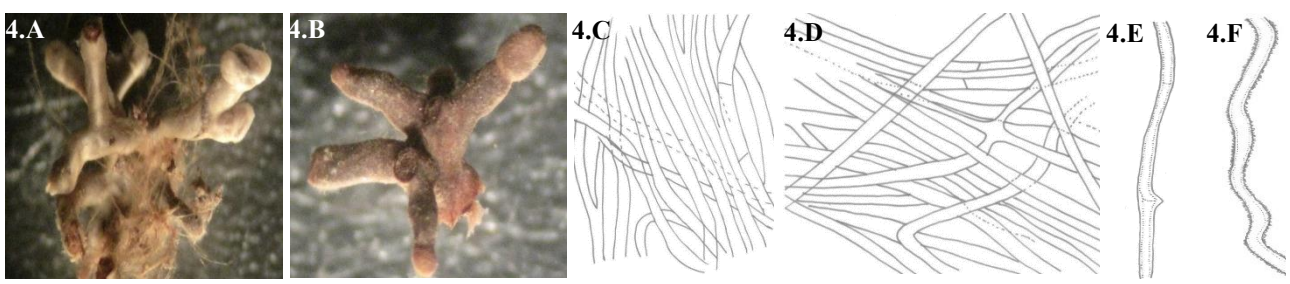

Fig. 4. A-F: Morpho-anatomical features of *Sebacina* sp. MHAFE-01 (A-B) ECM morphotypes of *Sebacina* sp. MHAFE-01 (C) Plectenchymatous Outer mantle (D) Plectenchymatous Inner mantle (E-F) Emanting Hyphae.

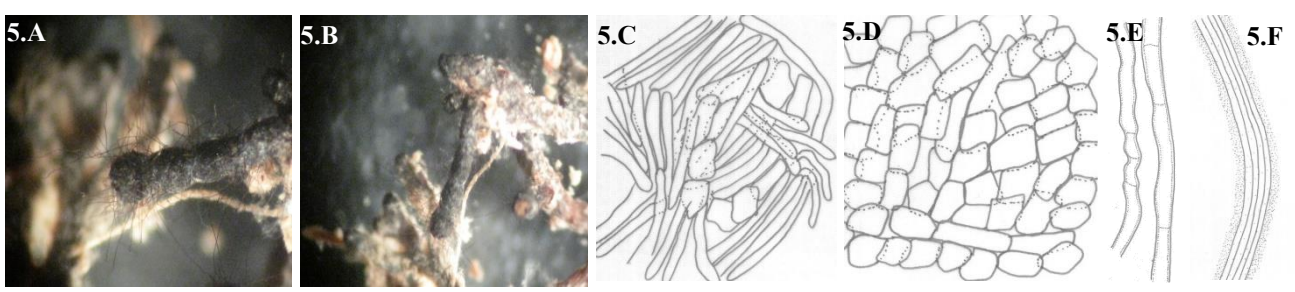

Fig. 5. A-F: Morpho-anatomical features of *Tomentella* sp. MHSTU-01 (A-B) ECM morphotypes of *Tomentella* sp. MHSTU-01 (C) Plectenchymatous Outer mantle (D) Pseudoparenchymatous Inner mantle (E) Emanating Hyphae (F) Rhizomorph.

Anatomical characters of mantle: Mantle transitionally plectenchymatous OUTER MANTLE plectenchymatous, some irregularly shaped rounded cells also intermingled with hyphae (mantle type H; Agerer, 2006), cells up to 5.2-9.4µm in length and 4.0-4.5µm in width. Hyphae up to 2.6-3.1µm in width, light brown in color, contents clear, hyphal junctions absent, matrix material not visible. INNER MANTLE pseudoparenchymatous, with hyphae intermingled with angular-shaped cells, up to 5.4-9.7µm in length and 4.2-4.5µm in width, pale brown in color, contents clear, hyphal junction absent, septa absent, matrix material not visible.

Anatomical features of emanating elements: EMANATING HYPHAE frequent, wavy to straight, up to 4.0-4.2µm in width, dark brown, contents clear, frequently septate, thick-walled, clamps absent, ornamentation absent, ramification absent, anastomoses absent.

RHIZOMORPHS present, light brown, aseptate, clampless hyphae, neatly aggregated together, contents clear, gelatinous material abundant on hyphae, up to 118-141.6µm in diameter with a restricted point of attachment.

***Tomentella* sp. MHSUB-01 Plate 01 (Figs. 6A-E)**

Morphological characters: ECTOMYCORRHIZAL SYSTEM simple to irregular, axis 15.0×22.0-0.5×1.0mm, UNRAMIFIED ENDS straight to club-shaped, 5.0×7.0-0.5×1.0mm, color of tips blackish brown while black at maturity, texture of system is coarse with a matte luster. No visibility of host tissue beneath the mantle sheath. EMANATING HYPHAE infrequent, RHIZOMORPHS present.

Anatomical characters of mantle: MANTLE pseudoparenchymatous, OUETR MANTLE pseudoparenchymatous, with tightly arranged rectangular cells (mantle type L; Agerer, 2006), up to 7.2-8.0µm in length while 7.7-7.8µm in width, light brown in color, contents clear, ornamentation absent, matrix material not visible. INNER MANTLE pseudoparenchymatous, cells up to 7.0-7.7µm in length and 7.5-7.8µm in width, contents clear, ornamentation absent, matrix material not observed.

Anatomical characters of emanating elements: EMANATING HYPHAE infrequent, straight, hyaline, up to 10.0-13.0µm in width, thin walled, moderately septate, clamped, ramification absent, contents clear, ornamentation absent. RHIZOMPRPHS abundant, off-white, numerous hyphae septate hyphae aggregated together, up to 180.5-182.3µm in width with the restricted point of attachment.

Molecular and Phylogenetic Identification of fungal taxa: From the living roots system of *A. nitida*, 12 fungal taxa were isolated, sequenced and BLAST searched for the closest match in GenBank (Table 1).

The distribution of these 12 phylotypes was based on the cladistic distribution of fungal taxa associated with *A. nitida*. The cladogram included ectomycorrhizal phylotypes distributed in 7 clades (Fig. 7).

In the Agaricales clade, we included 3 ectomycorrhizal fungal species from the genus *Inocybe* associated with this host tree. *Inocybe* sp. MHNIT-01 isolates (ENA16.42, ENA68.44, ENA76.41, 3ENA74.43 and 3ENA73.43) shared 97-100% analyzed genetic characters/nucleotides with each other and shared 93-95% analyzed genetic characters/nucleotides with similarity with *Inocybe nitidiuscula* (JF908249.1) while *Inocybe* sp. MHOBL-01.2ENA52.58 shared 93% of analyzed genetic characters/nucleotides with*Inocybe oblectabilis* (AM882831.2). These phylotypes/OTUs from Pakistan grouped in clade Agaricales with strong (92%) bootstrap support (Fig. 1).

Atheliales clade was represented by the genus *Amphinema*and *Tylospora*. From Pakistan, *Amphinema* was first time being reported from belowground community associated with *A. nitida*.All isolates of *Amphinema*sp. MHBYS-01 (3ENA89.48, ENA60.45, ENA52.48, 3ENA90.48 and ENA15.49) shared 100% of analyzed genetic characters with each other and 83% analyzed genetic characters/nucleotideswith *Amphinema byssoides* (AY219839.1) while *Tylospora*sp. MHTYL shared 99% of analyzed genetic characters with *T. fibrillosa* (HM1900017). The clade formed by Atheliales was not highly resolved by bootstrapping (54%) for the present analysis. Species of order Thelephorales grouped in two paraphyletic clades. Clade Thelephorales-I represented by *Pseudotomentella* species/isolates and Clade Thelephorales-II presented by *Tomentella* species. Both OUTs of *Pseudotomentella* sp. MHTRI (3ENA75.43 and 3ENA85.44) shared 98% of their analyzed genetic characters with each other and shared 89% analyzed genetic characters with *Pseudotomentella tristis* (AJ889968.1). Other clade of the Thelephorales (Clade-II) included only species of *Tomentella* and two OUTs from Pakistan clustered with strong (82%) bootstrap support. *Tomentella*sp. MHSTU.ENA77.41 shared 78% analyzed genetic characters with *T. stuposa* (EU819523.1) while *Tomentella* sp. MHSUB.2ENA09.40 shared 96% analyzed genetic characters with *T. subtestacea* (JQ711878.1).

Clade Sebacinales represented by only one phylotype (*Sebacina* sp. MHEPI-2ENA51.67) with strong (98%) bootstrap support from Pakistan. This morpho-phylotype shared 94.5% analyzed genetic characters with *Sebacina epigaea* (JQ665492.1).

Lastly, species of order Pezizales were also grouped in two clusters. *Helvella* sp. MHELA (2ENA08.40 and 2ENA07.40) shared 100% genetic characters with each other and 87% with *H. elastica* (AF335455.1). All phylotypes grouped together with strong bootstrapping (99%). Two morpho-phylotypes were represented with in the second cluster (Clade Pezizales-II). *Hydnobolites* sp. MHCER01 (ENA17.42) shared 82% genetic characters with *H. cerebriformis* (EU784272.1) and *Peziza* sp. MHMIC (3ENA88.45) shared 99% genetic characters with *P. michelii* (JF908562.1) and all phylotypes grouped together with strong bootstrap (99%) support (Fig. 01).

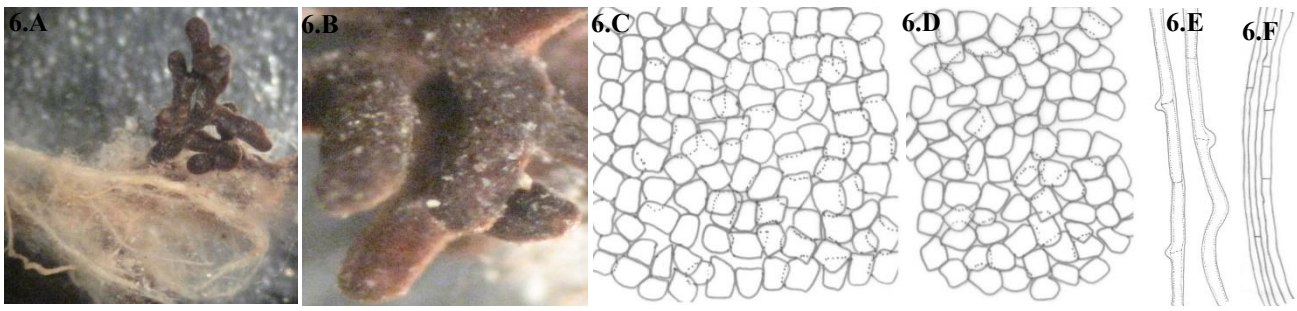

Fig. 6. A-F. Morpho-anatomical features of *Tomentella* sp. MHSUB-01 (A-B) ECM morphotypes of *Tomentella* sp. MHSUB-01 (C) Pseudoparenchymatous Outer mantle (D) Pseudoparenchymatous Inner Mantle (E) Emanating Hyphae (F) Rhizomorph.

Table 1. rDNA-ITS sequences from the below-ground mycoflora of *Alnus nitida* with their closest matches in the Genbank and their country of origin.

S. No.	Fungal species	Voucher No.	Accession No.	Closest match in GenBank	GenBank Accession No.	Origin country	Max. Identity (%)	Query coverage (%)
1.	*Amphinema* sp. MHBYS-01.	3ENA89.48	JN133916	*Amphinema byssoides*	AY219839.1	Canada	84%	99%
2.	Helvella sp. MHELA.	2ENA08.40	Awaiting	*Helvella elastica*	AF335455.1	Canada	92%	89%
3.	*Hydnobolites* sp. MHCER.	ENA17.42	Awaiting	*Hydnobolites cerebriformis*	EU784272.1	England	88%	90%
4.	*Inocybe* sp. MHNIT-01	ENA16.42	Awaiting	*Inocybe nitidiuscula*	JF908249.1	USA	95%	99%
5.	*Inocybe* sp. MHNIT-02	.ENA76.41	Awaiting	*Inocybe nitidiuscula*	JF908249.1	USA	94%	93%
6.	*Inocybe* sp. MHOBL-01	.2ENA52.58	Awaiting	*Inocybe oblectabilis*	AM882831.2	Sweden	95%	99%
7.	*Peziza michelii*	3ENA88.45	JN836749	*Peziza michelii*	JF908562.1	USA	99%	100%
8.	*Pseudotomentella* sp. MHTRI-01	.3ENA75.43	Awaiting	*Pseudotomentella tristis*	AJ889968.1	Denmark	93%	87%
9.	Pseudotomentella sp. MHTRI-02	.3ENA85.44	Awaiting	*Pseudotomentella tristis*	AJ889968.1	Denmark	100%	88%
10.	*Sebacina* sp. MHEPI	.2ENA51.67	Awaiting	*Sebacina epigaea*	JQ665492.1	Germany	95%	98%
11.	*Tomentella* sp. MHSTU.	ENA77.41	Awaiting	*Tomentella stuposa*	EU819523.1	USA	79%	93%
12.	*Tomentella* sp. MHSUB.	2ENA09.40	Awaiting	*Tomentella subtestacea*	JQ711878.1	Canada	96%	99%

Discussion

The Himalayan Alder's ectomycorrhizae were first documented by Ashraf *et al.*, (2012) from Pakistan. They reported *Peziza michelii* as ectomycorrhizal with this photobiont. Recently, Tapwal *et al.*, (2021) reported *Ramaria stricta* as ectomycorrhizal with this host. McBurney *et al*., (2017) reported 22 ectomycorrhizal fungi from *Alnus* sp. (*A. rubra*). Kilpeläinen *et al*., (2019) reported *Amanita muscaria*, *Hebeloma*, *Laccaria laccata*, *Lactarius aspideus*, *Leccinum aurantiacum* and *Paxillus involutus* were reported as ectomycorrhizal with another *Alnus* sp. (*A. incana*). Present investigation documents 11 ectomycorrhizae of Himalayan alder. These were characterized using the conventional and the modern molecular methods and were identified.

Amphinema sp. MHBYS-01 Ectomycorrhizae (closely matched with *Amphinema byssoides*) are being described and illustrated first time in association with *Alnus nitida*. Previously ectomycorrhizae of *A. byssoides* were reported with *Picea abies* (Agerer, 1987-1995; Berg, 1989; Danielson & Pruden, 1989; Haug & Pritsch, 1992; Ingleby *et al.*, 1990; Weiss, 1988, 1989, 1990; Weiss & Agerer, 1988), *Pinus* (Fassiand de Vecchi, 1962; Haug *et al.*, 1994; Raidl, 1997), and *Quercus ilex* (Montecchio *et al.*, 2002). With *Picea abies*, it formed monopodial-pinnate ramification, with *Pinus* dichotomous, with *Quercus* irregularly pinnate to dichotomous-like. While with *Alnus nitida*, *Amphinema* sp. MHBYS-01, forms simple to irregular system with dichotomously branched unramified ends. Besides ramification, the mantle anatomy also differed when compared with morphotypes from other photobionts. *Picea abies*, *Pinus* and *Quercus ilex* morphotypes have plectenchymatous mantle organization while *Alnus nitida* has transitionally pseudoparenchymatous mantle organization. Despite this, *Amphinema* sp. MHBYS-01 has hyphae that lacked septa and clamps and also lacked rhizomorphs, young tips skin-colored, oxblood red at maturity, frequent emanating hyphae. The fruitbody of this fungus was previously found growing on the stump of *Pinus wallichiana*in forests of Murree (Ahmad, 1972). Phylogenetically, *Amphinema* sp. MHBYS-01 grouped with *A. byssoides* with strong (96%) bootstrap support. All these morpho-anatomic and molecular phylogenetic differences are significant for delimiting *Amphinema* sp. MHBYS-01 from *A. byssoides*. *Amphinema* seems to generalist mycorrhizal forming

fungus as it is part of many ecologically different belowground communities (Kjøller *et al.*, 2008; Taylor & Finlay, 2003; Qian *et al.*, 1998; Taylor & Brand, 1992). *Picea abies* and *Pinus cembra* were also found to form ectomycorrhizal associations with *Amphinema* (Margit *et al*., 2010). It was also found with *Pinus sylvestris* (Aučina *et al.*, 2007).

Ectomycorrhiza of the *Helvella* sp. MHELA-02 (closely matched with *Helvella elastica*) had light brown to beige color. Ectomycorrhizal system is characterized by simple to irregularly dichotomous with bulbous to club-shaped unramified ends.

Ectomycorrhiza of the *Helvella* sp. MHELA-02 (closely matched with *Helvella elastica*) can be compared with *Helvella* sp. Ho-TS601 which seems a close relative of *Helvella* sp. MHELA-02. Ectomycorrhizae of *Helvella* sp. Ho-TS601 was described by Tedersoo *et al.*, (2006) and was characterized by orange-brown to red-brown morphotypes and whitish tips when young. The mantle organization was cellular with large spherical to subepidermoid cells, without emanating hyphae. The species in genera *Helvella* are treated with unresolved trophic status (Hansen and Pfister 2006; Læssøe & Hansen, 2007). There was no previous molecular evidence about its trophic status. Tedersoo *et al.*, (2007a) designated this genus as ectomycorrhizal on the basis of stable isotopic analysis. O'Donnell *et al.*, (1997), Hansen & Pfister (2006) and Tedersoo *et al.*, (2006a) provided molecular (rDNA-ITS based) evidence about the mycorrhizal status of this group. On a similar rDNA-ITS barcoding basis, *Helvella* sp. MHELA-02 from Pakistan was assigned as mycorrhizal and its mycorrhizae are first time described and illustrated from Asia. This phylotype was recently reported as part of different belowground ectomycorrhizal communities of the Himalayan Moist temperate forests of Pakistan (Hanif, 2012).

We did not find any published description of ectomycorrhizae of *Inocybe nitidiuscula* (closely match of *Inocybe* sp. MHNIT-01) ectomycorrhizae to compare its morpho-anatomic features with *Inocybe* sp. MHNIT-01. Phylogenetically, this phylotype clustered with *I. nitidiuscula* sequences and thus was identified as a sister species to *I. nitidiuscula*. Previously, it was reported as ectomycorrhizal in the association of seedlings of *Pyrola chlorantha* (Hynson *et al.*, 2013).

Sebacina sp. MHAFE-01 (closely matched with *Sebacina incrustans*) was found growing as mycorrhizal with roots of *A. nitida*. Their mycorrhizal morphotypes differed significantly from *Sebacina incrustans*. Urban *et al.*, (2003) described mycorrhizae of *Sebacina incrustans* in the association of *Picea*. These ectomycorrhizae have monopodial-pyramidal ramification and lack rhizomorphs. The color of their mycorrhizal roots ranged from yellow pale cream or faintly yellowish to yellowish or white; mantle organization plectenchymatous, hyphae arranged net-like, repeatedly and squarrosely branched. On the other hand, *Sebacina* sp. MHAFE-01 ectomycorrhizae have simple to the irregular ectomycorrhizal system with simple to bulbous unramified ends. Members of *Sebacinales* are part of many ectomycorrhizal communities (Takahide *et al.*, 2007; Selosse *et al.*, 2007; Martin *et al.*, 2009; Setaro *et al.*, 2006; Morris *et al.*, 2008; Smith *et al.*, 2007). Hanif (2012) also reported *Sebacinales* from Himalayan Moist Temperate Forests of Pakistan.

The mycorrhizal interactions in *Sebacinales* may have arisen from an ancestral endophytic habit by specialization (Weiß *et al.*, 2011). Considering their proven beneficial influence on plant growth and their ubiquity, endophytic *Sebacinales* may be a previously unrecognized universal hidden force in plant ecosystems.

In the present work, two more ectomycorrhizal morphotypes of the genus *Tomentella* are described and illustrated morpho-anatomically and phylogenetically. Ectomycorrhizal morphotypes of *Tomentella* sp. MHSTU-01 (closely matched with *Tomentella stuposa*) are simple to irregular with simple unramified ends with black tips, coarse texture of system with a matte luster. Phylogenetically, *Tomentella* sp. MHSTU-01 clustered with *Tomentella stuposa* (Fig. 01). Ectomycorrhizal morphotypes of *T. stuposa* were described previously (Agerer *et al.*, 2002; Jakucs *et al.*, 2005) with *Pinus* sp. These were brown in color and had monopodial-pinnate or monopodial-pyramidal ramification, rhizomorphs presence present, mantle pseudoparenchymatous with angular cells, bearing mounds of roundish cells (type K), emanating hyphae presence. *T. stuposa* ectomycorrhizae were also observed in association with *Salix reticulata* and *Dryas octopetala* communities (Ryberg *et al.*, 2009) and many other ectomycorrhizal communities (Cline *et al.*, 2005; Tedersoo *et al.*, 2003; Smith *et al.*, 2007; McCormick *et al.*, 2004). Hanif *et al.*, (2012) described and illustrated isolates of *Tomentella* spp. with *Cedrus deodara* from Pakistan.

Phylogenetically, *Tomentella subtestacea* (closely matched) appeared as sister species of *Tomentella* sp. MHSUB-01 (Fig. 01). Both these phylotypes differed significantly on the basis of shared analyzed genetic characters and rDNA-ITS genetic divergence. Ectomycorrhizae of the *Tomentella* sp. MHSUB-01 differed from *T. subtestacea* in having a simple to the irregular ectomycorrhizal system with straight to club-shaped unramified ends. The tips of the system are blackish brown while black at maturity, texture of the system is coarse with a matte luster. While, *T. subtestacea* has a monopodial-pinnate ectomycorrhizal system with straight unramified ends (Jakucs & Agerer, 2001). Previously *T. subtestacea* was reported it ectomycorrhizal from terrestrial Orchids below ground community (McCormick *et al.*, 2004).

The existence of the diverse types of ectomycorrhizal fungal species with *A. nitida* growing in the Himalayan moist temperate forests of Pakistan indicates its preference for more mycobiont partners. To complete the below-ground picture of the ectomycorrhizal communities associated with *A. nitida*, more comprehensive sampling would be required.

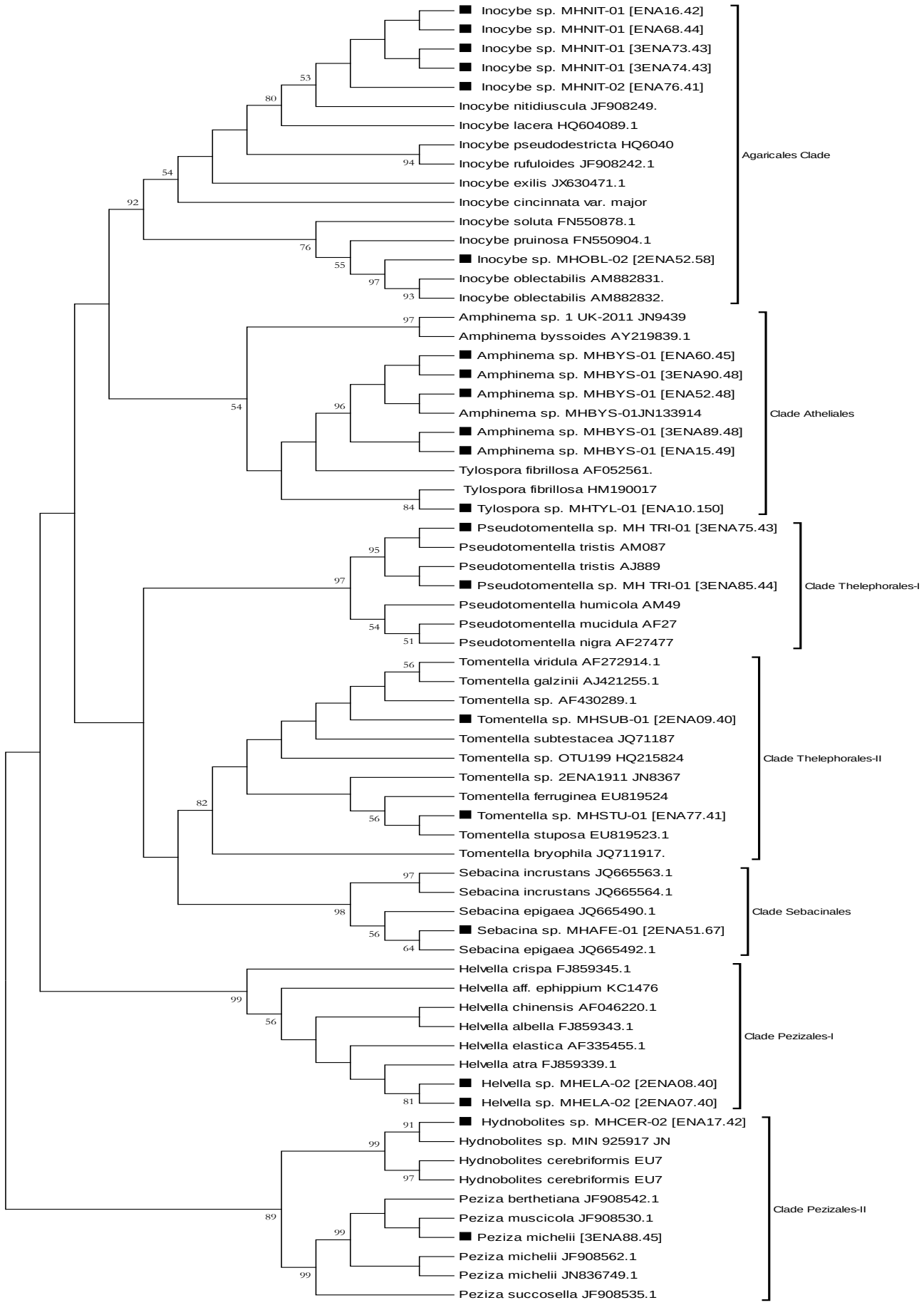

Fig. 7. Phylogenetic tree for Ectomycorrhizal MOTUs associated with *Alnus nitida* and rDNA-ITS sequences retrieved from GenBank.

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