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Peziza michelii and its ectomycorrhizae with *Alnus nitida* (*Betulaceae*) from Pakistan

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Abstract — *Peziza michelii* and its ectomycorrhizae with *Alnus nitida* are characterized morphologically and molecularly by nrDNA ITS1-5.8S-ITS2 sequence analyses. Both the fungus and its morphotype are new records for Pakistan.

Key words — operculate asci, *Pezizales*, phylogeny

Introduction

Peziza Dill. ex Fr. (*Pezizales, Pezizaceae*) is a large, widely distributed heterogeneous genus represented by 104 species (Kirk et al. 2008). Its trophic status ranges from saprobic to mycorrhizal (Hansen et al. 2001).

Identification of pezizalean mycorrhizae using morphological or conventional methods has remained difficult since they lack hyphal strands, connecting apothecium, and diagnostic morphotypes. Generally pezizalean ECM (ectomycorrhizae) possess a thin pseudoparenchymatous mantle, infrequent emanating hyphae, and rhizomorphs, and clamp connections are lacking (Agerer 1991, 2001). At the present time molecular tools are quite helpful for ECM identification, and the ECM of many *Peziza* species have been confirmed using such tools. Species identified as mycorrhizal with deciduous trees include *Peziza depressa* Pers., *P. michelii*, *P. ostracoderma* Korf, *P. succosa* Berk., and *P. succosella* (Le Gal & Romagn.) M.M. Moser ex Aviz.-Hersh. & Nemlich (Tedersoo et al. 2006).

Betulaceae is represented in Pakistan by two genera, *Alnus* and *Betula* (Naisr 1975), for which there are no previous reports about ectomycorrhizal status from Pakistan. Previously, *P. michelii* has been reported as ectomycorrhizal with *Alnus* sp., *Picea abies* (Tedersoo et al. 2009), *Betula* sp. (Tedersoo et al.

2008), *Fagus orientalis* (Bahram et al. 2012) *Tsuga canadensis* (McLenon-Porter 2008), and *Tilia* sp. (Lang et al. 2011). Mycorrhizal associations of *Alnus* with *P. michelii* are confirmed molecularly in this study.

Ahmad et al. (1997) and Ashraf & Khalid (2012) have reported 89 pezizalean taxa (including nine *Peziza* species) from Pakistan, and ascocarp surveys in Pakistan account for eight known to be ectomycorrhizal. Molecular characterization of *Pezizales* and their ectomycorrhizae in Pakistan is in progress. Our study, which documents association of *P. michelii* with *Alnus nitida* (Spach) Endl. in Asia for the first time, is the first attempt at morphological and molecular characterization of ascomycete morphotypes from Pakistan.

Materials & methods

Ascomata were collected and photographed; necessary data was recorded in field and specimens were dried with the help of a fan heater. Ascomata were rehydrated and free hand sections were made in the laboratory. Microscopic (morpho-anatomical) characters were noted at 10× and 40× and drawings were made with the help of camera lucida.

Soil under *A. nitida* was sampled and washed to isolate, characterize, and identify ECM. The selected morphotypes were characterized morphologically following Agerer (2001) and have been deposited in Herbarium of Botany Department (LAH), University of the Punjab in Lahore.

DNA was extracted from dried ascomata and morphotypes using Extract N. Amp.™ Plant kit (SIGMA). The nrDNA ITS1–5.8S–ITS2 region was amplified using fungal specific primers pairs (ITS1F/ITS4) following manufacturer's protocol with denaturation at 94°C for 4 min, followed by 35 cycles of 45 sec at 94°C, 45 sec at 54°C, and 1 min 30 sec at 72°C, and a final extension at 72°C for 2 min. After purification, the PCR products underwent bidirectional sequencing by Macrogen (South Korea). GenBank ITS sequences were BLAST-searched for sequence comparison and identification. Selected sequences were aligned using Clustal W and corrected manually. All ambiguous insertions and deletions were removed prior to further analyses. Sequences have been accessioned in GenBank.

Results

Alnus nitida roots were found to be colonized by *Peziza michelii*. The BLAST search of the ITS sequences from *P. michelii* ascomata and morphotypes associated with *Alnus nitida* obtained in the present study showed a 99% identity and 98% query coverage match with *P. michelii* from Denmark (DQ200839.1). The identity percentage was calculated at 96.3, showing a 97% identity and minimum divergence of 1.5 and 0.8 compared with *Peziza michelii* (DQ200838.1 and DQ200839.1) respectively (Plate 1). The topology and identification of *P. michelii* was confirmed by the neighbor-joining method

and maximum likelihood method (Plates 2–3). Both analyses place *P. michelii* with *P. succosa* and *P. succosella* as sister clade.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		61.4	85.8	81.2	60.7	61.2	51.6	68.3	58.7	52.4	59.4	62.1	62.1	56.5	62.9	83.9	1
2	0.7		49.7	46.0	36.2	34.9	28.0	42.0	37.2	33.4	38.5	42.6	54.3	34.8	38.9	48.2	2
3	0.6	0.4		95.5	70.9	70.2	62.1	64.9	66.8	61.6	62.8	54.3	50.4	65.9	63.2	97.0	3
4	0.8	0.8	0.2		71.0	68.6	66.2	60.5	62.9	65.6	58.5	55.5	51.7	64.8	59.2	96.3	4
5	26.3	29.7	26.6	26.8		94.5	83.4	84.4	65.5	69.2	64.6	60.2	55.5	67.6	63.4	72.4	5
6	26.6	28.6	26.9	27.5	3.5		82.4	85.9	66.9	67.5	66.2	58.5	53.7	67.5	64.5	70.3	6
7	28.9	33.8	28.2	27.6	7.0	7.1		76.6	62.2	70.0	60.7	58.1	54.3	62.9	56.7	63.4	7
8	25.4	27.2	28.2	29.5	5.0	5.8	6.1		61.8	59.9	65.5	67.0	61.1	60.7	69.2	62.5	8
9	32.7	28.2	31.4	32.2	31.6	31.2	34.5	33.2		75.1	79.3	65.2	57.7	78.0	72.6	64.5	9
10	37.7	34.0	37.1	36.6	33.9	33.7	32.8	36.6	16.8		71.2	70.7	63.2	77.1	70.2	63.4	10
11	33.3	31.5	36.4	38.4	30.9	30.7	34.8	30.2	18.7	20.9		65.3	59.1	75.0	71.6	60.7	11
12	39.5	37.9	41.4	41.4	37.4	38.8	38.3	37.6	20.9	21.1	24.7		75.3	68.0	73.6	53.3	12
13	34.7	32.5	34.9	34.9	31.5	32.9	34.4	30.6	23.5	21.5	25.0	16.8		60.8	66.2	49.4	13
14	34.4	30.9	33.2	33.6	32.5	33.6	34.1	34.3	18.1	18.5	22.5	15.8	17.8		76.1	66.5	14
15	36.2	36.0	37.8	39.2	35.9	35.9	35.9	34.2	20.9	20.7	24.0	17.5	15.7	15.4		61.6	15
16	0.4	1.5	0.8	1.5	26.3	27.3	28.4	28.7	32.2	36.2	37.0	41.1	34.5	33.3	37.7		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 Peziza_michelii_ENA38845_Pakistan
2 Peziza_michelii_FR852088.1_Iran
3 Peziza_michelii_DQ200839.1_Denmark
4 Peziza_michelii_DQ200838.1_Denmark
5 Peziza_infossa_DQ974817.1_USA
6 Peziza_cf._succosa_EU819417
7 Peziza_succosella_DQ200841.1_Denmark
8 Peziza_succosa_DQ200840.1_Denmark
9 Peziza_phyllogena_AY789329.1_USA
10 Peziza_ostracoderma_JN002180
11 Chromelosporium_carneum_FJ7911
12 Peziza_stuntzii_FJ268642.1_USA
13 Cazia_flexiascus_EU846309
14 Peziza_depressa_DQ200837
15 Mycoclelandia_arenacea_Q231745
16 Peziza_michelii_TA116_Pakistan

Plate 1. Percent divergence and identity calculated by comparing sequence pairs reconstructed by MegAlign (DNASTAR).

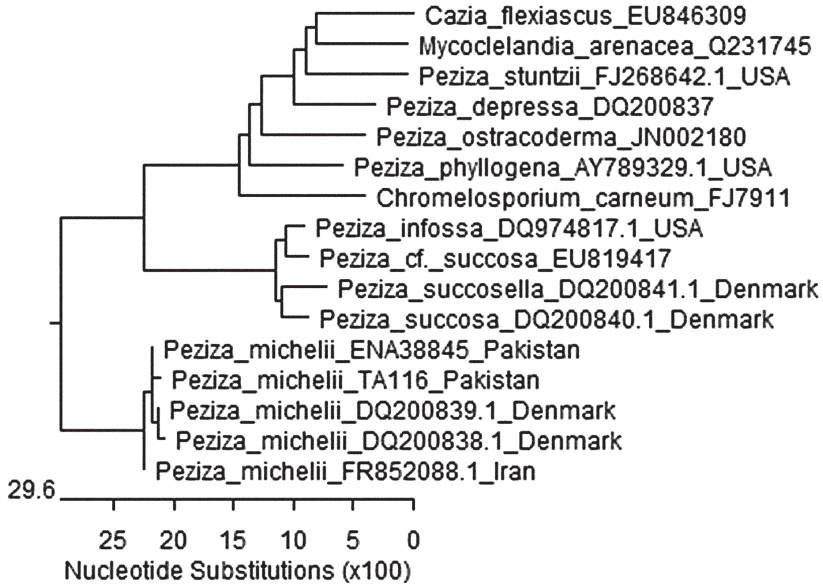

Plate 2. Phylogenetic tree based on ITS1-5.8S-ITS2 nucleotide sequences adjusted to 722 bases and constructed by the neighbor-joining method using MegAlign. The scale indicates the percentage of base difference (% divergence). Sequence data for phylogenetic analysis were taken from the GenBank nucleotide sequence database for *Peziza*, *Cazia*, *Chromelosporium*, and *Mycoclelandia*.

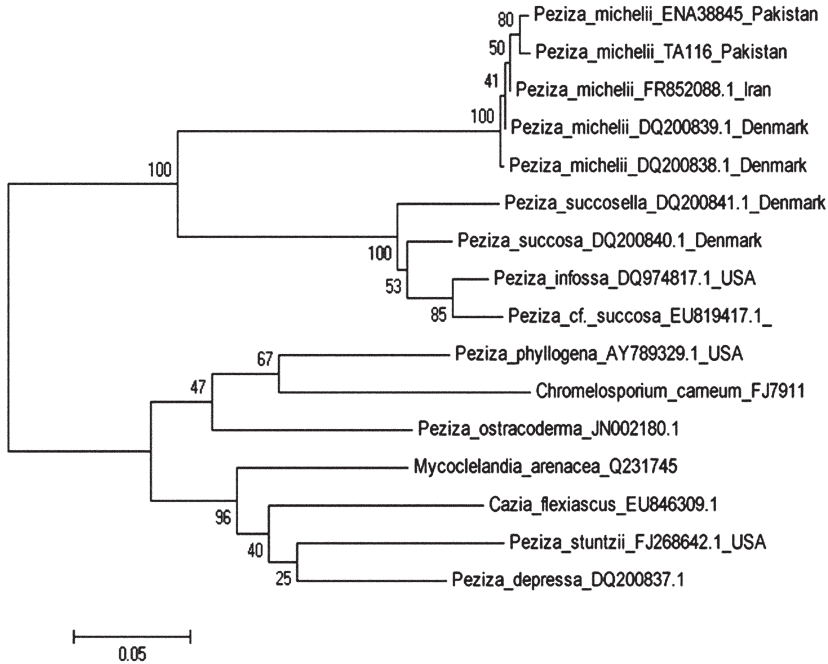

Plate 3. Molecular Phylogenetic analysis was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (–6466.0969) is shown. Bootstrap values are shown against each branch. Initial tree(s) for the heuristic search were obtained automatically: maximum parsimony method was used when common sites numbered <100 (or <1/4 total sites); otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The analysis involved 16 sequences.

Taxonomy

Peziza michelii (Boud.) Dennis, Brit. Cup Fungi & Allies: 15 (1960) Plate 4

Apothecia cupulate, medium-sized, diam. up to 5 cm, deeply concave, margins entire, but turning inwards, somewhat raised and torn, hymenium reddish brown with purplish tinge, smooth, outer surface yellow to yellowish brown, sessile to subsessile, solitary. Hymenium 290 µm thick. Asci cylindrical, operculate, unitunicate, 8-spored, uniseriate, strongly amyloid at apex, but diffusely blue along length, slightly narrowing near base, 260–278(–300) × 13–17 µm. Ascospores uniseriate, ellipsoid, biguttulate, ornamented, 15–17(–19) × 9–10 µm, ornamentation verrucose to warted: which appear elongated, irregularly and widely spaced, 0.3–0.7µm high. Paraphyses long, slender, septate, with light brownish content, straight, 2–3 µm wide, slightly thick up to 5–6 µm at tip. Excipulum: Ectal excipulum a textura globulosa to angularis, 100–160 µm thick, cells hyaline to slightly brown, thin–walled, diam. 10–22

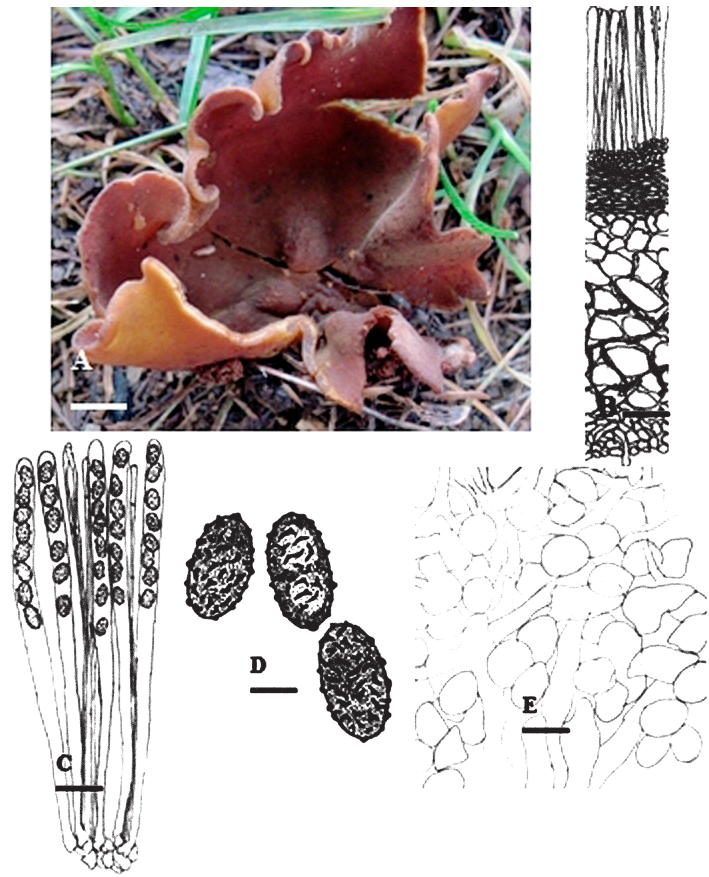

Plate 4. *Peziza michelii*: A. Apothecium. B. Apothecial section showing hymenium, sub-hymenium, medullary, and ectal excipulum. C. Part of hymenium, showing asci with ascospores and paraphyses. D. Ascospores. E. Excipulum. Scale bars: A = 0.5 cm; B = 90 µm; C = 30 µm; D =6 µm; E = 80 µm

µm; medullary excipulum a textura globulosa, 500 µm thick, larger sub-globose thin-walled cells, 22–40 µm diam., interspersed with numerous interwoven delicate hyphae ≤8 µm diam.

Material examined: **PAKISTAN**: **Khyber Pakhtunkhwa**, Khanspur, Helipad, Himalayan moist temperate forests, 2575 m (8205 ft) altitude, solitary, on ground, mossy substrate, 21 Aug. 2010, T. Ashraf TA-116 (LAH 210810; GenBank JN836748).

Morphological description **Plate 5**

Ectomycorrhizal system simple, axis 4–5 × 1.0–1.3 mm. Unramified ends rounded to bent, bifurcate, 2–3 mm long and 0.8–1.2 mm in diam.,

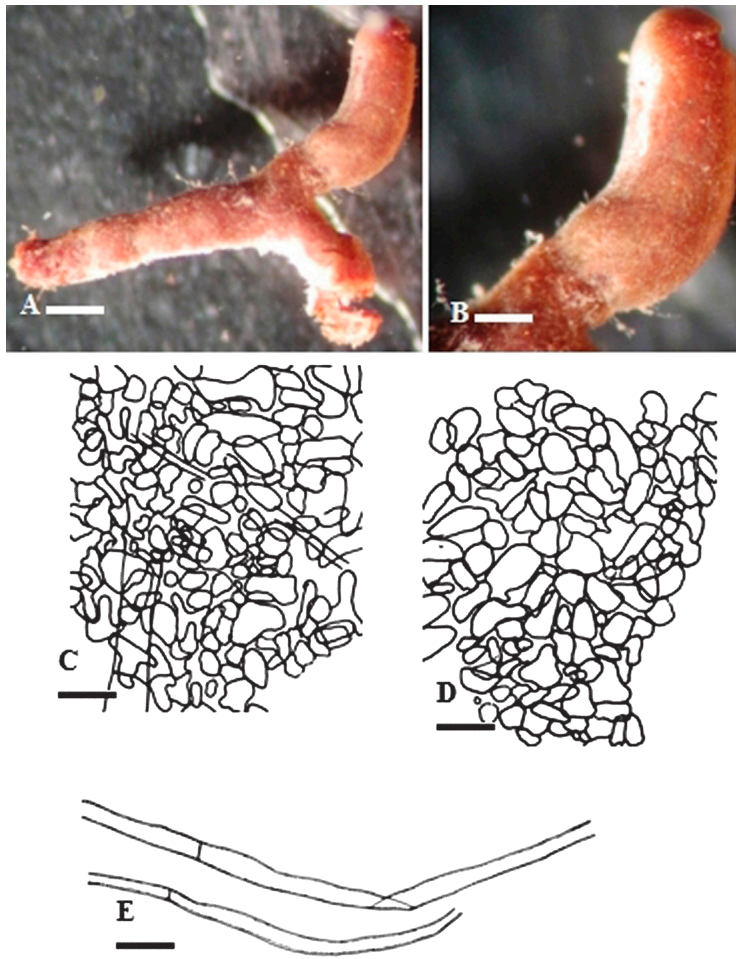

Plate 5. ECM of *Peziza michelii*: A–B. Habit. C. Pseudoparenchymatous outer mantle. D. Pseudoparenchymatous inner mantle. E. Emanating hyphae. Scale bars: A=0.5 mm; B= 0.5 mm; C–D = 2 µm; E= 1.5 µm.

younger tips creamy white, older tips brown to black. Texture of system felty to velvety with matte luster, host tissue not visible under the sheath. Rhizomorph absent. Emanating hyphae rare, straight.

Mantle pseudoparenchymatous in all layers, Outer Mantle pseudoparenchymatous with irregular (ovoid to epidermoid) cells, hyphal cells infrequent and inconspicuous, hyphae without clamps, light yellowish, no cell

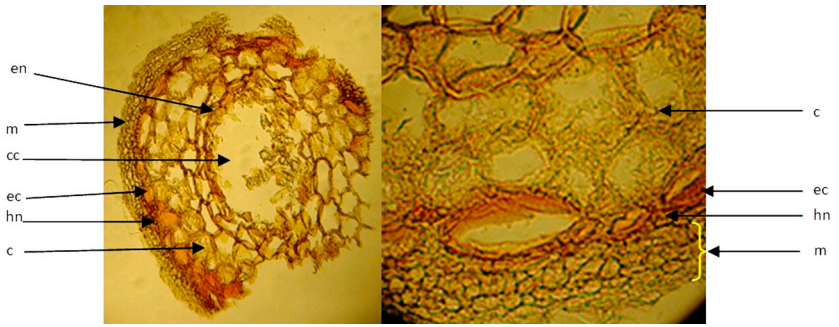

Plate 6. Cross section of *Alnus nitida* ectomycorrhizal root: m = mantle, ec = ectoderm, c = cortex, en = endoderm, cc = central cylinder, hn = hartig net.

contents visible, cells 14–15 × 10–14 µm. Inner Mantle pseudoparenchymatous, cells colorless to light yellowish, cells roundish to irregular in shape, no matrix material observed, cells 13–15 × 11–14 µm.

Mantle thickness 36 µm, ≤ 5 layers thick, discernible, cells rectangular to tangentially oval and/or tangentially elliptical. Hartig net paraepidermal, followed by large cortical cells, endodermis and central cylinder (Plate 6)

Material examined: **PAKISTAN**: **Khyber Pakhtunkhwa**, Khanspur, Helipad, Himalayan moist temperate forests, 2575 m (8205ft) altitude, roots of *Alnus nitida*, 15 May 2010, M Hanif ENA38845 (LAH 150509; GenBank JN836749).

Discussion

The present work is the first report of *Peziza michelii* from Pakistan. This species can be found frequently in Himalayan moist temperate forests of Pakistan. Phylogenetically, *P. michelii* clusters with the similar species from USA, Iran, and Denmark.

Tedersoo et al. (2006) studied the morphotypes of *P. michelii* morphologically and used molecular markers to identify the mycobiont. They found these ectomycorrhizae to be solitary, rare, in small clusters, minute, conspicuous, and yellow green to olive green. This finding differed from the present report and morphotypes associated with *Alnus nitida* from Pakistan. Those morphotypes we studied are simple, creamy white with brown to black older tips, and lacking a rhizomorph. The anatomical features of the mantle surface also differ slightly in both morphotypes, with a mantle surface varying from smooth to finely granular in the morphotype reported by Tedersoo et al. (2006) versus the *A. nitida* morphotype, which is felty to velvety with matte luster with almost equal thickness (2–4 hyphal layers vs. 5 hyphal layers). Other mantle features in both studies also varied. The morpho-anatomic features of both morphotypes

of *Peziza michelii* may vary because of interaction of mycobiont with different photobionts.

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